A Structural and Biochemical Study of the Corneal Stroma

Thesis

A Structural and Biochemical Study of the Corneal Stroma

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To Freda and Elisabeth

Abstract

It is thought that the structural organisation of the cornea, in particular the uniform size and spacing of its constituent collagen fibrils, is a major factor responsible for its transparency. The roles of the various components of the corneal stroma in the maintenance of its structure are not currently well understood, although it is thought that proteoglycans and the glycosylation of collagen may be involved in the control of fibril diameter.

Protein components were removed from the corneal stroma, under mild and harsh conditions of extraction, and analysed using polyacrylamide gel electrophoresis and other biochemical techniques. A range of 0.15M NaCl-soluble proteins and proteoglycans could be removed under mild extraction conditions, including a proteoglycan of very high molecular weight which has not been reported elsewhere. Under harsh conditions of extraction, when 1% SDS was added to the extracting solution, an additional glycoprotein, designated GP135, was removed.

The effect of the extraction procedures on the structure of the cornea was studied using electron microscopy and X-ray diffraction. Electron microscopy showed increasing disruption to the ultrastructure of the cornea with more harsh extraction methods, and indicated that material had been removed from the gap region of the collagen fibrils. Loss of the interfibrillar X-ray diffraction pattern following extraction also indicated disruption of the ordered structure, and electron density profiles obtained from the meridional diffraction pattern suggested removal of material from the gap region.

The coincidental removal of GP135 from the cornea with the removal of material from the gap region of the collagen fibrils indicated that it might be located here.

Transparency measurements from extracted corneas showed that the loss of structural order produced by these methods might not be the most important factor in the loss of transparency for corneas at physiological hydration. Also, the transparency of highly hydrated corneas is dependent on the type of solution they are immersed in.

Acknowledgements

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Abbreviations

BSA	Bovine serum albumin
CEC	Critical electrolyte concentration
GAG	Glycosaminoglycan
GuCl	Guanidinium chloride
DNA	Deoxyribosenucleic acid
PG	Proteoglycan
KSPG	Keratan sulphate proteoglycan
DSPG	Dermatan sulphate proteoglycan
PKS	Proteokeratan sulphate
PDS	Proteodermatan sulphate
KS	Keratan sulphate
DS	Dermatan sulphate
CS	Chondroitin sulphate
EDTA	Ethylenediaminetetracetic acid
SDS	Sodium dodecylsulphate
PEG	Polyethylene glycol
PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic acid/Schiff stain
PBS	Phosphate-buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TCA	Trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
M_r	Relative molecular mass
v/v	volume/volume
w/v	weight/volume

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Chapter 1

Introduction

This thesis is concerned with the nature and roles of the proteins removed from the corneal stroma under a variety of extraction conditions. In this chapter the structure of the cornea and its relationship to other ocular tissues will be considered. This will be followed by a detailed description of the constituents of the corneal stroma; collagens, proteoglycans, glycoproteins and various other components. This information has been collected together from a wide variety of literature from different sources. Theories accounting for the transparency of the cornea will be introduced. Finally, the objectives of this thesis and a summary of the methods used to achieve them will be discussed.

1.1 Anatomy of the eye

Figure 1.1 is a diagrammatic representation of the eye showing the arrangement of its components. The cornea is continuous with the sclera, together they form a tough protective coating for the more delicate optical components of the eye. The retina is the light-sensitive layer on the posterior surface of the eye. In order to obtain good visual acuity, the components in front of the retina, i.e. the cornea, lens, aqueous humour and vitreous humour, need to be transparent: opacity in any of these components leads to defective vision. The eye also needs to be able to focus light effectively onto the retina to form a sharp image. The cornea and lens form the focussing

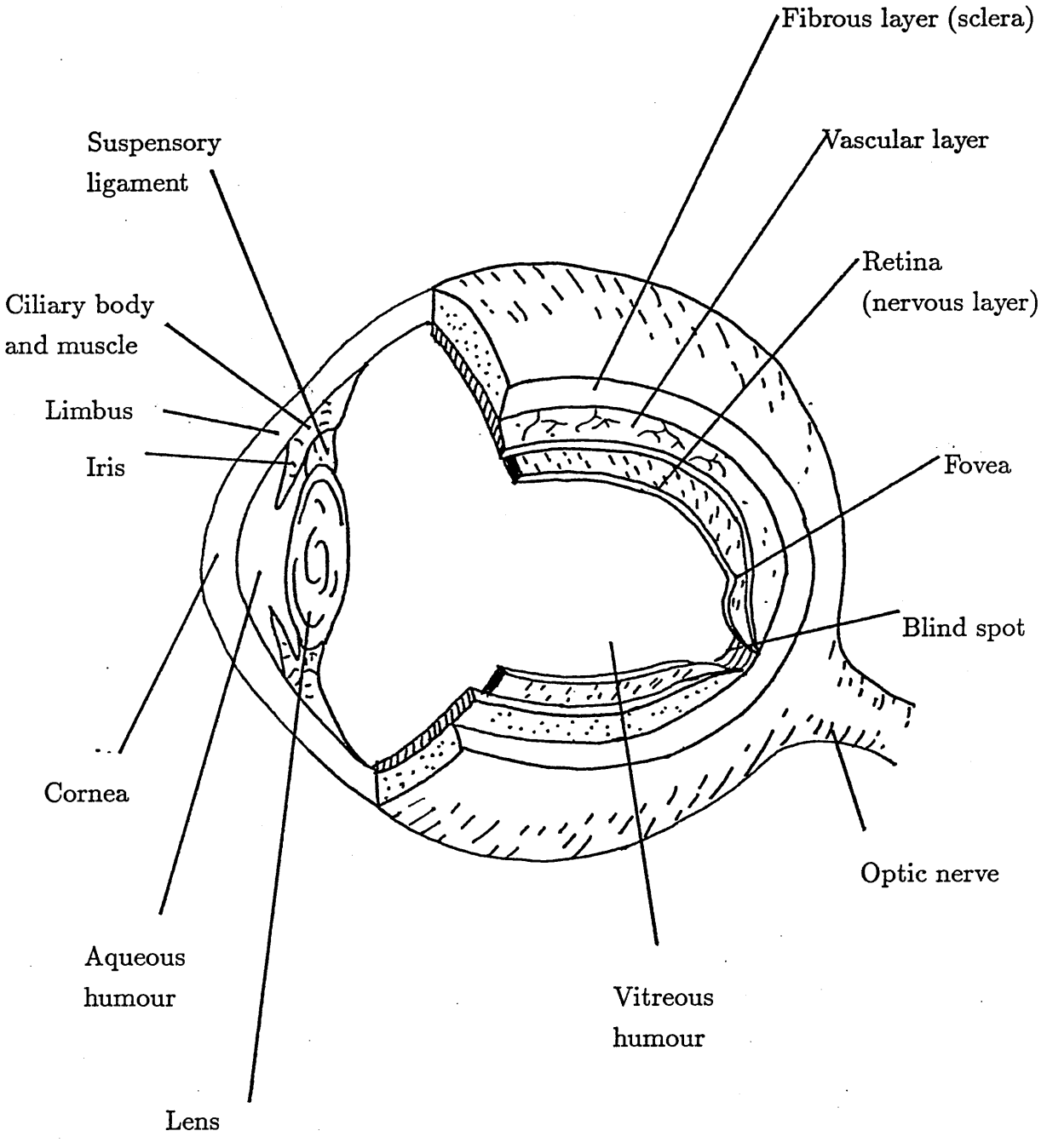


Figure 1.1 Anatomy of the eye.

system of the eye.

1.2 Properties and structure of the cornea

From the foregoing description of the anatomy of the eye it is clear that the cornea needs to be:

1. tough to withstand any external damage and also the intraocular pressure exerted by fluid in the eye.

2. transparent in order to allow light to enter the eye and reach the retina.

3. curved, and with a refractive index such that a large part of the focussing is performed by it; typically cornea accounts for two-thirds of the focussing power of the eye. The remainder of the focussing necessary to produce an image on the retina is done by the lens.

The adult human cornea is typically 11mm in diameter, and 0.52mm thick at its central portion, increasing slightly towards the limbus. Figure 1.2 is a diagrammatic representation of the structure of the cornea, showing its five constituent layers. From the anterior surface to the posterior these are; epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium. There are many review articles describing the features of each constituent layer in detail, eg Maurice, 1969, so these will not be dealt with here.

1.3 Detailed composition of the stroma

In most species water constitutes 75–80% of the fresh weight of the cornea. For comparison between different corneas, this is generally expressed as a hydration value, H, which is calculated as shown:

$$\mathrm{H} = \frac{(\text{wet wt.}) - (\text{dry wt.})}{(\text{dry wt.})}$$

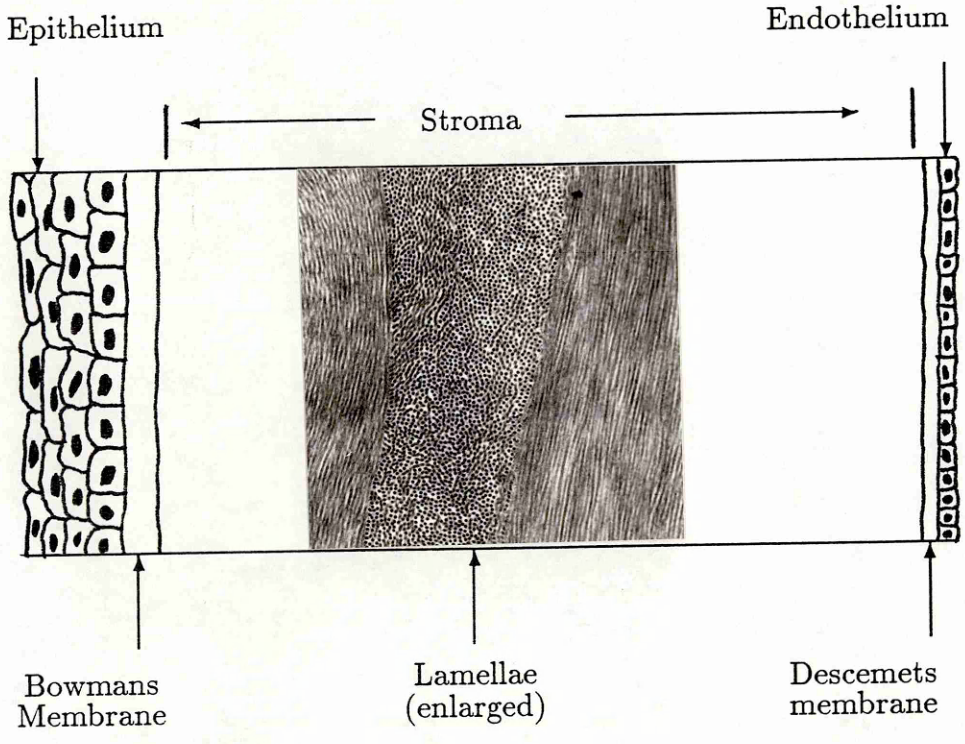


Figure 1.2 The structure of the cornea, showing its constituent cellular and extracellular layers.

Measurements of the hydration of fresh human cornea range from 3.5 [Fatt, 1978] to 3.68 [Mishima, 1968]. The physiological hydration of corneas from different species does not vary appreciably from those of human cornea [Gyi, 1988].

Table 1.1 shows the chemical composition of the corneal stroma. The original dry weight figures [Maurice, 1969] give a value of 65.2% for the proportion of collagen, whereas more recent figures give 90–95% as the proportion of collagen [Winterhalter, 1988]. The reason for this discrepancy lies in the recent discovery that there are many different types of collagen which vary in their primary structure. The original analysis included 22.7% by weight of 'other proteins', whereas in the later analysis the majority of these are now classified as collagens, with only 1% of the dry weight being attributed to 'other proteins'. Originally, the proportion of collagen would

Components	% wet wt.	% dry wt.(†)	% dry wt.(‡)
Water	78	-	-
Collagen	15	65.2	90–95
Other proteins (including PG core proteins)	5	22.7	1
Glycosaminoglycans	1	4.6	5
Salts	1	4.5	-

Table 1.1 Composition of the corneal stroma. Values for the % wet wt. are taken from Maurice (1969), these refer to bovine cornea; the % dry wt.(†) figures are calculated from these. The % dry wt.(‡) figures are given by Winterhalter (1988), these refer to human cornea.

have been estimated on the basis of the hydroxyproline content of the tissue, which gives a measure of the amount of triple-helical collagen in the tissue. It is now known that some collagens contain substantial protein extensions which are not triple-helical. These extensions consist of protein in a globular arrangement, which do not contain hydroxyproline residues. However, the new figure of 90–95% for the proportion of collagen may be too high, as the method used to calculate it could well have overestimated

the amount of collagen in the tissue. This is discussed further in the section which deals with type VI collagen.

Other constituents of the corneal stroma include proteoglycans, glycoproteins, other proteins and salts. The dry weight figure for glycosaminoglycans is very similar in the two analyses, no figure for the percentage dry weight of salts is given by Winterhalter.

1.3.1 The presence and roles of different collagen types in the corneal stroma

Table 1.2 shows the proportions of the currently known collagen types obtained from analysis of adult human cornea. A notably high value for the proportion of type VI collagen is given. This will be discussed in the section dealing with type VI collagen. The various collagen types are described in more detail below.

Collagen type	% of total corneal dry wt.
I	52
III	1
IV	1
V	8
VI	30
VII	1
VIII	1

Table 1.2 Collagen types expressed as a percentage of total corneal dry weight in adult human cornea [Winterhalter, 1988].

Type I collagen

Type I collagen is a major component in many connective tissues such as skin and tendon, and is the predominant fibril-forming collagen of the corneal stroma. Three polypeptide chains, each of M_r 100 000, combine in a triple-helical arrangement to form the collagen molecule (see figure 1.3). A

definitive feature of this collagen, or the triple-helical portion of any other collagen, is the presence of the repeating amino acid sequence Gly-X-Y in the constituent polypeptide chains, where X and Y are commonly proline or hydroxyproline. The presence of glycine every third residue allows the three chains to twist around each other closely; stabilization of the molecule by interchain hydrogen bonding can then occur.

Further analysis of the polypeptide constituents of type I collagen has shown that it is composed of two different polypeptides, designated α1(I) and α2(I). The type I molecule has been found to consist of one α2(I) and two α1(I) chains [Piez *et al.*, 1963]. After rotary shadowing individual molecules can be seen in the electron microscope as thin, flexible rods approximately 300nm in length [Hall and Slayter, 1959].

Type II collagen

This is the major collagenous component of hyaline cartilage. Three identical α1(II) polypeptide chains of M_r 100 000 combine to form a triple-helical molecule in the same way as type I collagen. The molecules associate laterally to form banded fibres in the same way as type I collagen, but these tend to be of much smaller diameter, typically 10nm in cartilage. Type II collagen has not been found in the human or bovine cornea, however, it is present in developing avian cornea [Linsenmayer *et al.*, 1982] and in mouse cornea [Harnisch *et al.*, 1978].

Type III collagen

This collagen has a similar molecular structure to type I, consisting of a rod-like triple-helical molecule composed of three identical α1(III) chains of M_r 100 000. Type III collagen has been found in fibrils separate from type I [Fleischmajer *et al.*, 1981], and also in association with type I [Henkel and Glanville, 1982]. The physiological function of a particular connective tissue relates directly to its production of fibril-forming collagens (types I–III and V), although the exact role of each is not currently understood.

Type IV collagen

Early X-ray studies of basement membrane indicated that it contained collagen [Pirie, 1951]; this collagen was subsequently designated type IV collagen [Kefalides, 1973], as it was clearly different from the other interstitial collagens known at the time. In cell culture, two different kinds of polypeptide chains are synthesised to produce type IV collagen, α1(IV) with M_r 180 000 and α2(IV) with M_r 165 000. These combine, probably as heterotrimers i.e. $(\alpha 1(IV))_2\alpha 2(IV)$ to produce monomeric type IV collagen which is observed in the electron microscope as a single triple-helical strand with a small globular extension on one end. The monomers can associate in one of two ways (see figure 1.3) to produce either a network arrangement, or a regular polygonal structure such as is seen in Descemet's membrane in the cornea [Sawada, 1982].

Type V collagen

Type V collagen is similar to type I in that it contains a triple-helical section of about the same length as the type I molecule, with non-collagenous propeptides attached at each end [Bächinger *et al.*, 1982]. Current estimates show that it comprises 8% of the corneal collagen; localisation in cornea indicates that it is associated with the type I fibres, and that the type I collagen masks the type V to some extent [Linsenmayer *et al.*, 1986]. If types I and V are present within the same fibre, they could be evenly distributed throughout or associate in separate regions within the fibre, either of which could influence such factors as fibril diameter and tensile strength of the fibres.

Type VI collagen

This collagen is likely to be of major importance in the corneal stroma as current estimates indicate that it may comprise up to 30% of the total collagen. It was first isolated from pepsin digests of aortic intima [Chung *et al.*, 1976] as a large, disulphide-bonded complex with a unique

amino a[illegible]l composition. Upon reduction, a characteristic peptide pattern cou[illegible]be seen after gel electrophoresis, with peptides of M_r~50 000. Subseque[illegible]ly, similar complexes were isolated from other tissues such as liver [illegible]ojkind *et al.*, 1979], placenta [Furoto and Miller, 1980], skin [Laurain e[illegible]*l.*, 1980] and uterus [Abedin *et al.*, 1982].

The int[illegible]t form of the collagen was isolated from cell culture medium, where its c[illegible]stituent polypeptide chains were found to be rich in carbohydrate. T[illegible]three chains which form the monomer appear to be non-identical, th[illegible]α1(VI) and α2(VI) chains with M_r 140 000 and the α3(VI) chain with M[illegible]200 000 [Trüeb and Winterhalter, 1986]. Amino acid analysis of intact [illegible]VI) chains from different tissues shows a glycine content of less than 20%[illegible]hich is much lower than that expected for a triple-helical molecule.

Type VI col[illegible]en is thus a hybrid molecule consisting of part triple-helix and part globul[illegible]protein. This can be seen very clearly from rotary shadowing pictures o[illegible]ype VI molecules, where these appear dumbell-shaped with two globul[illegible]domains of approximately equal size connected by a 105nm long tripl[illegible]elical section. The monomers associate to form high molecular weight [illegible]nplexes as shown in figure 1.3, these probably give rise to the banded mic[illegible]ibrils of 110nm periodicity seen in various tissues.

In table 1.2 the[illegible]roportion of type VI collagen is given as 30%. This figure is arrived at [illegible]owing pepsin digestion of the tissue, and comparison of the amount of typ[illegible]VI fragments to type I collagen extracted. This ratio is then used to calcu[illegible]e the proportion of type VI collagen in the tissue. However, the cornea[illegible]sed to obtain these results were from 80 year old individuals [Zimmern[illegible]n *et al.*, 1986], where the amount of extractable type I collagen would b[illegible]xpected to be very much reduced, due to extensive cross-linking. Thus, ca[illegible]lating the proportion of type VI collagen by this method would lead to a[illegible]rtificially high value. This would also be reflected in the high figure of 90-[illegible]% for the dry weight of total collagen.

In cornea, some [illegible]udies indicate that type VI collagen is present in microfibrils [illegible]ich are independent of the type I fibrils

[von der Mark *et al.*, 1984,Linsenmayer *et al.*, 1986], although there may be some interaction between the two [Alper and Amenta, 1986], whereas others have found type VI in regular association with the type I fibres [Menasche, 1988].

The function of this collagen is not currently known. Possible roles include that of an attachment protein for cells to their substratum, and of adapter molecule between collagenous and globular proteins [Trüeb and Bornstein, 1984]. The latter would seem more likely in the case of corneal stroma as the relatively small number of cells present would not require the high level of type VI which is found. Its role as an adapter molecule would make type VI collagen an ideal candidate as part of an organisational system for the type I fibrils and proteoglycans in corneal stroma.

Type VII collagen

This collagen is a major component of anchoring fibrils which attach epithelial cells to their underlying stroma in various connective tissues. The type VII molecule is composed of three polypeptides of M_r~350 000 which form a triple-helical centre section with globular extensions on each end (see figure 1.4). Two of these molecules form an antiparallel dimer, and this basic unit condenses laterally to form fibrils. These fibrils then interact with other collagens in the basement membrane, notably type IV, to form an extended anchoring network. In human cornea this network is located at the epithelial edge of Bowman's membrane [Burgeson, 1987].

Type VIII collagen

Type VIII collagen was first discovered in endothelial cell culture medium, hence its original designation EC, endothelial collagen [Sage *et al.*, 1979]. Three polypeptides, each of M_r 180 000, associate to form a molecule composed of three collagenous domains, separated by non-collagenous linking regions (see figure 1.4). In the cornea, type VIII collagen is a major component in Descemet's membrane, where it is deposited in a hexagonal

lattice arrangement [Labermeier and Kenney, 1983,Kapoor *et al.*, 1985]. The function of type VIII collagen is thought to be that of maintenance of endothelial cell integrity and differentiated phenotype [Sage and Bornstein, 1987].

Type IX collagen

Burgeson and Hollister (1979) first discovered type IX collagen in pepsin digests from cartilage. It is composed of three polypeptide chains, α1(IX), α2(IX) and α3(IX), which associate to form a molecule comprising three collagenous and four non-collagenous domains (see figure 1.4); chondroitin sulphate has also been shown to be present, attached to the α2(IX) chain [Huber *et al.*, 1986]. Its presence in developing avian primary corneal stroma has been demonstrated by Fitch *et al.*, (1988), who propose that the function of the molecule is to form intermolecular cross-bridges between type I collagen fibrils, thereby resisting swelling of the matrix during early developmental stages.

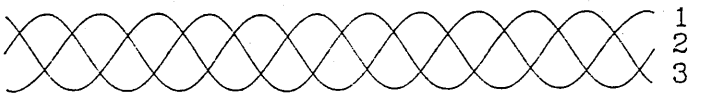
Other collagen types

Type X collagen is produced by chondrocytes [Gibson *et al.*, 1981], types XI and XII by cartilage [Burgeson and Hollister, 1979]. Collagen types XIII and XIV have also recently been discovered. To date, none of these have been reported in the cornea.

1.3.2 Formation of collagen fibrils

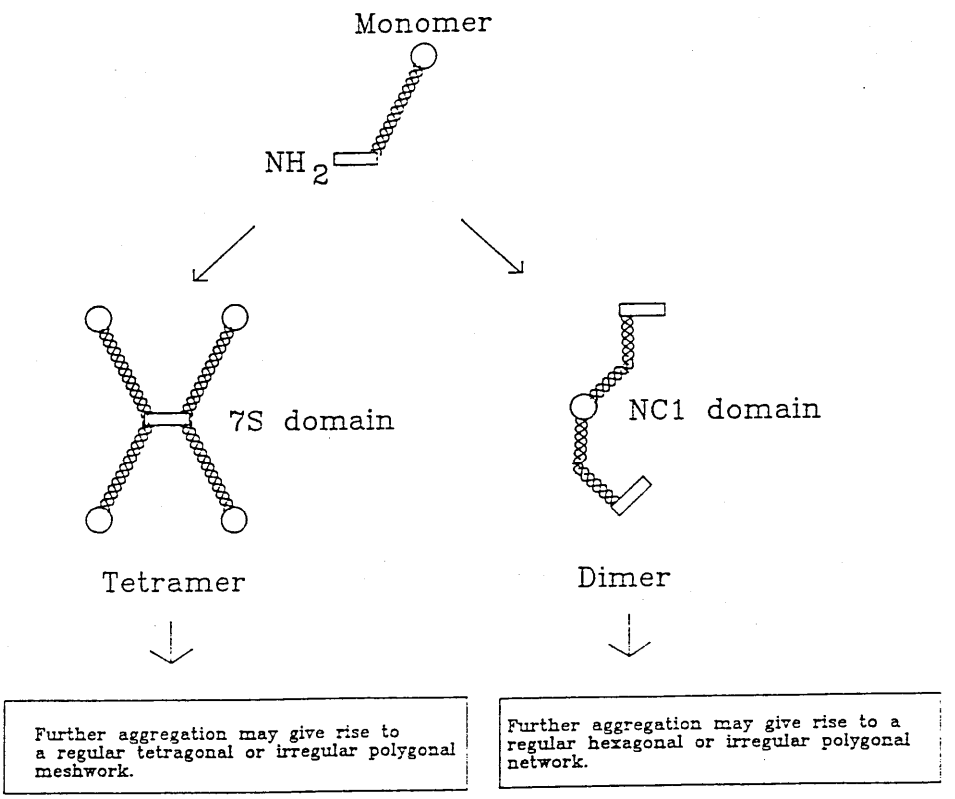
In cornea, as in many other connective tissues, fibrils formed are predominantly of type I collagen. Individual collagen molecules align side-by-side in a staggered arrangement as shown in figure 1.5 (page 14). This arrangement gives rise to a step in electron density along the fibril [Tomlin and Worthington, 1956], from which the staggered arrangement of the molecules could be deduced [Hodge and Petruska, 1963]. The gap/overlap pattern produced by the molecular packing arrangement is pe-

Structure of Types I, II, III and V Collagen

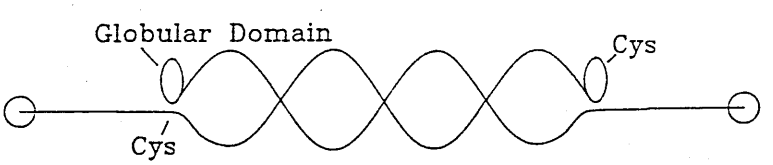


Types I, II, III, and V collagen all have a similar molecular structure, consisting of three polypeptide chains arranged in a triple helix.

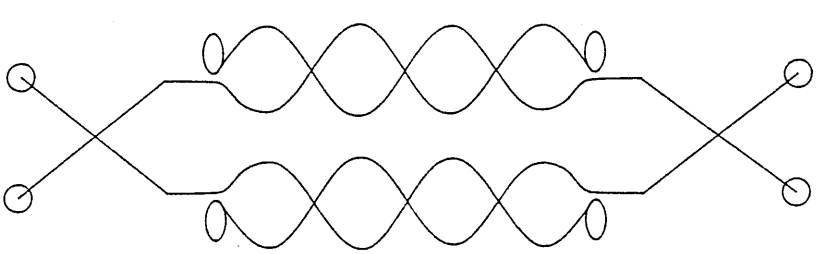
Structure of Type IV Collagen



Structure of Type VI Collagen



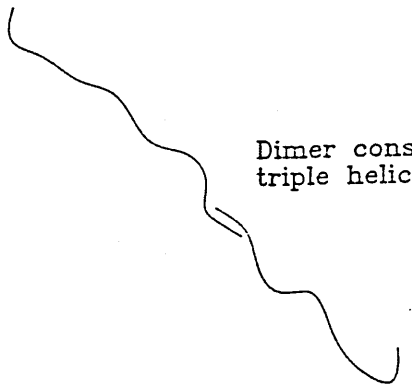
Dimeric form of Type VI Collagen



Tetrameric form of Type VI Collagen

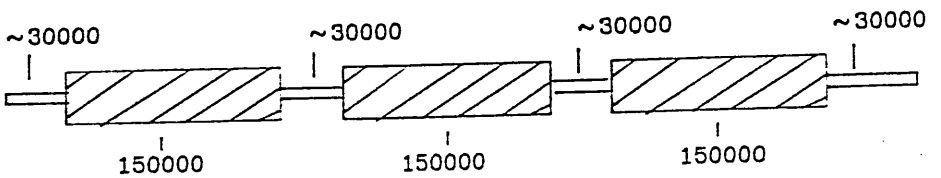
Figure 1.3 Model of collagen types I–VI.

Structure of Type VII Collagen



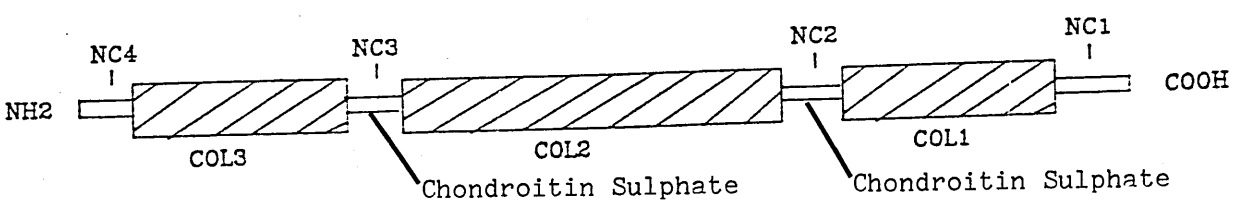
Dimer consisting of two Type VII Collagen triple helical domains.

Structure of Type VIII Collagen



Type VIII Collagen consists of three polypeptide chains each of Mr 180000 which associate to form a molecule of Mr 540000. Each chain has three 'cassettes' of Mr 50000 each, which associate to form the triple-helical sections of the Type VIII molecule.

Structure of Type IX Collagen



The extended model for the structure of Type IX Collagen indicates that the molecule consists of three collagenous triple-helical domains (COL1,2,3) and four non-collagenous domains (NC1-NC4).

Figure 1.4 Model of collagen types VII-IX.

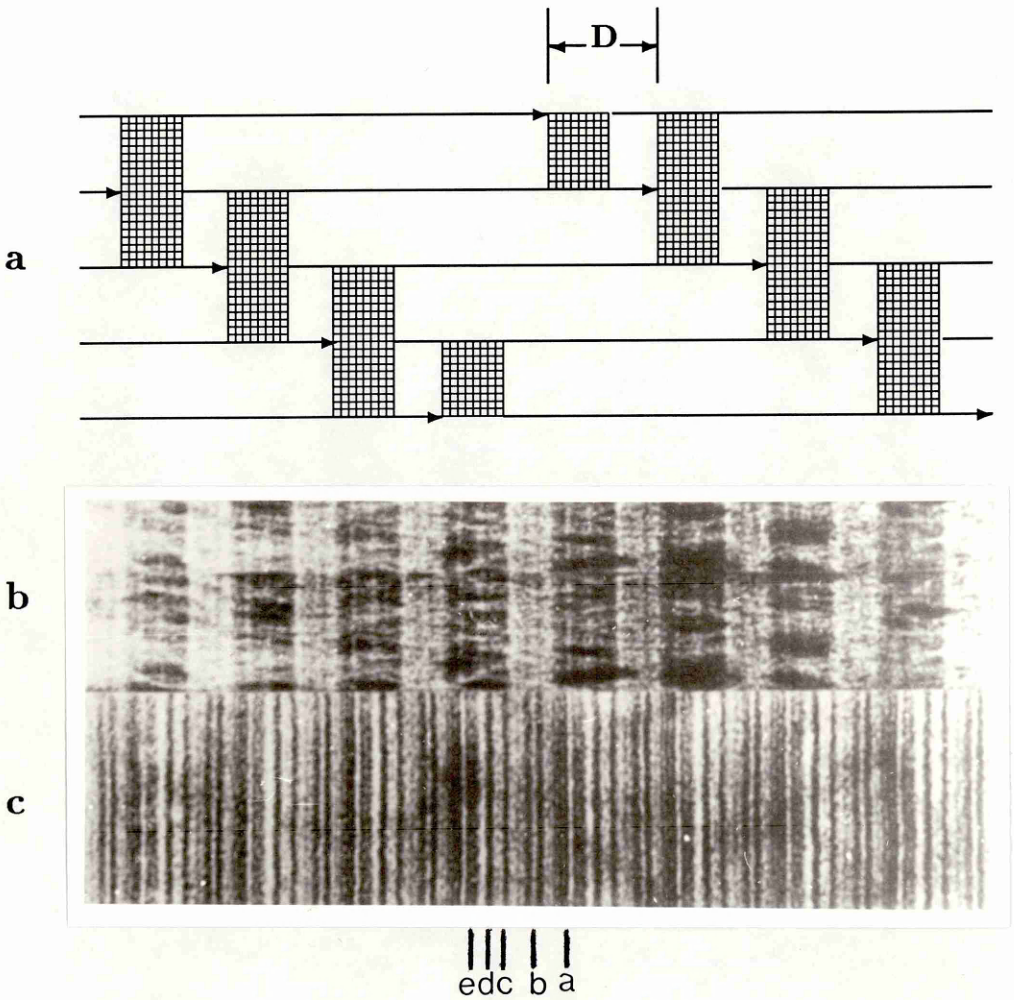


Figure 1.5 The formation of collagen fibrils and the electron microscopic banding pattern which it gives rise to.
a: Diagram of the arrangement of collagen molecules in a fibril. The arrows represent individual molecules. The cross-hatched areas show the position of the gaps in the fibril.
b: Negative staining pattern of tendon collagen, aligned with diagram a to show how the structure of the fibril gives rise to the banding pattern.
c: Positive staining pattern of tendon collagen. Charged residues are aligned side-by-side to produce the characteristic positive staining pattern in tendon collagen.

riodic, giving rise to an axial repeat in cornea of 65nm as shown by low-angle X-ray diffraction of wet specimens, and up to 64nm by electron microscopy, depending on the method of fixation used [Marchini *et al.*, 1986]; this repeat is referred to as the D-period.

A recent study of the formation of collagen fibrils, and the effect of proteoglycan preparations on their formation *in vitro*, has been made by Birk and Lande (1981), who used type I collagen obtained from rabbit cornea and sclera. Their work shows that fibrils from corneal collagen form 6–7 times more slowly than those from scleral collagen, and that proteoglycans derived from both cornea and sclera retarded collagen fibril formation. Examination of the collagens used in this study showed that lysine hydroxylation and hexose content were both higher in corneal compared to scleral collagen.

1.3.3 Electron microscopic staining of collagen

Type I collagen fibrils can be stained negatively using sodium phosphotungstate at neutral pH. Under these conditions stain collects in those areas of the collagen fibrils where it is able to penetrate, producing a dark band, and is excluded from areas where amino acid side chains have a high 'bulkiness' (average cross-sectional area), producing a light band [Tzaphlidou and Chapman, 1986]. In type I collagen fibres found in tendon this gives rise to the characteristic banding pattern as shown in figure 1.5. In cornea the negative banding pattern is not as clearly defined as in tendon, stain appears to be excluded from the gap zone to a large extent, which has led to the suggestion that additional material is present at the gap zone which inhibits negative stain penetration. Positive staining of fibres using phosphotungstic acid and uranyl acetate confirms the unidirectionality of the molecules within the fibre [Meek and Holmes, 1983]. The pattern produced in this case arises from the alignment of acidic and basic residues across the fibre, which take up stain thus giving rise to a series of transverse bands. These are referred to as bands a to e, using the notation of Hodge and Schmitt, (1960). Axial locations within the

D period can therefore be identified with respect to this banding pattern [Chapman, 1974]. The stain distribution in the bands can change under certain conditions if the charged residues are altered, e.g. during fixation [Meek and Chapman, 1985].

1.3.4 X-ray diffraction of collagen

X-ray diffraction can be used to study tissues which exhibit regularity of structure over a limited size range. It has two major advantages over electron microscopy,

1. Tissues can be studied in the wet, unfixed state, thus avoiding the difficulties which arise from the processes necessary for electron microscopy, such as fixation, drying and embedding. Analysis of the X-ray diffraction pattern thus provides information about the tissue in its physiological state, and no corrections need be made for tissue shrinkage.

2. The X-ray diffraction pattern is an averaged pattern over the area of tissue covered by the X-ray beam. Many electron micrographs need to be studied in order to be sure that the results seen are generally representative of the tissue, and are not due to a local difference in the tissue. The X-ray beam automatically averages over the area of tissue it covers, thereby giving a representative analysis for that area. This can have its disadvantages if there are highly localised differences in the structure, as it is not possible to infer these from an averaged picture.

In the cornea, the structure of the collagen fibrils and the regularity with which other components associate with the fibrils gives rise to a characteristic X-ray diffraction pattern. Figure 1.6 shows a typical diffraction pattern from bovine cornea. There are two components to this pattern [Goodfellow *et al.*, 1978], as seen in figures 1.6a and 1.6b.

a

b

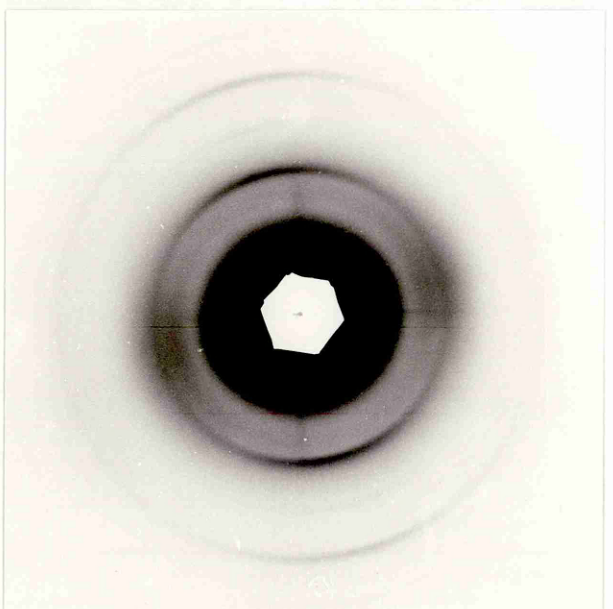


Figure 1.6 Typical X-ray diffraction patterns from bovine cornea.
To obtain these patterns the direction of the X-ray beam is perpendicular to the surface of the corneas examined.
a. The equatorial diffraction pattern. This is a ring, the diameter of which gives a measure of the most common interfibrillar spacing in the cornea.
b. The meridional diffraction pattern. This is a series of rings, the relative intensities of which can be used to compute the axial electron density of the collagen fibrils.

The equatorial diffraction pattern

This pattern, shown in figure 1.6a, arises from the regular side-to-side packing of the collagen fibrils. It is circular in bovine cornea, indicating that collagen fibrils are oriented in all directions equally in the plane of the cornea. The diameter of this ring may be used to calculate the most common interfibrillar spacing in the cornea. The width of the ring is related to the range of interfibrillar spacings present in the cornea.

The meridional diffraction pattern

This pattern, shown in figure 1.6b, arises from the axial electron density profile of the collagen fibrils. It is composed of a series of rings (orders of reflection) of varying intensities. The relative intensities of the different orders can be used to compute the axial electron density of the collagen fibrils, with finer detail being obtained if more orders can be included in the calculation. In work described in this thesis at least nine orders are used, together with a smoothing function [Meek *et al.*, 1981a], to reduce termination artefacts which can occur if too few orders are used. The axial electron density thus obtained can be compared with that of collagen from other tissues, e.g. tendon, to see where differences in electron density occur within a D-period.

1.3.5 The structure and type of proteoglycans in the cornea

Proteoglycans are hybrid molecules consisting of a protein core to which are attached long chains of highly sulphated repeating disaccharides, namely glycosaminoglycans; short chains of neutral sugars may also be attached to the protein core. In contrast, glycoproteins consist of a protein core to which are attached short chains of neutral sugars, which may be branched. Proteoglycans from different connective tissues vary in size and composition depending on their function; those from cartilage tend to be large, consisting of a core protein with a M_r of more than 200 000, with 100 attached

chondroitin sulphate (CS) chains, 30 keratan sulphate (KS) chains and 50–60 glycosidically linked oligosaccharides [Heinegård *et al.*, 1985a]. Association of these large proteoglycans with hyaluronate produces complexes with a M_r of approximately 100 million, which have the shock- absorbing properties necessary for cartilage function.

Figure 1.7 shows the chemical structures of the repeating disaccharide units of keratan sulphate, chondroitin-4-sulphate and dermatan sulphate. Under physiological conditions, the carboxyl and sulphate groups on the sugars would be ionized, thus producing highly negatively charged molecules. This feature contributes to the function of the proteoglycans in connective tissues, as it produces highly hydrated, space-filling molecules. In cornea, chondroitin sulphate is actually a hybrid molecule consisting of a mixture of chondroitin-4-sulphate and a small proportion of dermatan sulphate, hence its designation DS/CS [Scott, 1988].

In cornea there are currently four known populations of proteoglycans, all of which have core proteins with a M_r in the region of 45 000–50 000. The dermatan sulphate proteoglycans (PDSI and PDSII) contain 1 or 2 dermatan sulphate/chondroitin sulphate (DS/CS) chains [Heinegård *et al.*, 1985b]: The keratan sulphate proteoglycans (PKSI and PKSII) contain one keratan sulphate side chain with a M_r which can vary between 4 000 and 20 000 [Axelsson and Heinegård, 1978]. It is the variation in length of the attached glycosaminoglycan chains which gives rise to the size polydispersity found in these proteoglycans.

1.3.6 The location of proteoglycans associated with corneal collagen

Using a specific enzyme digestion method, Scott and Haigh (1985) have located keratan sulphate proteoglycan (KSPG) at the a and c bands of the collagen fibril in rabbit cornea, and dermatan sulphate proteoglycan (DSPG) at the d and e bands. Application of the critical electrolyte concentration method, whereby specificity of the proteoglycan stain Cupromeronic

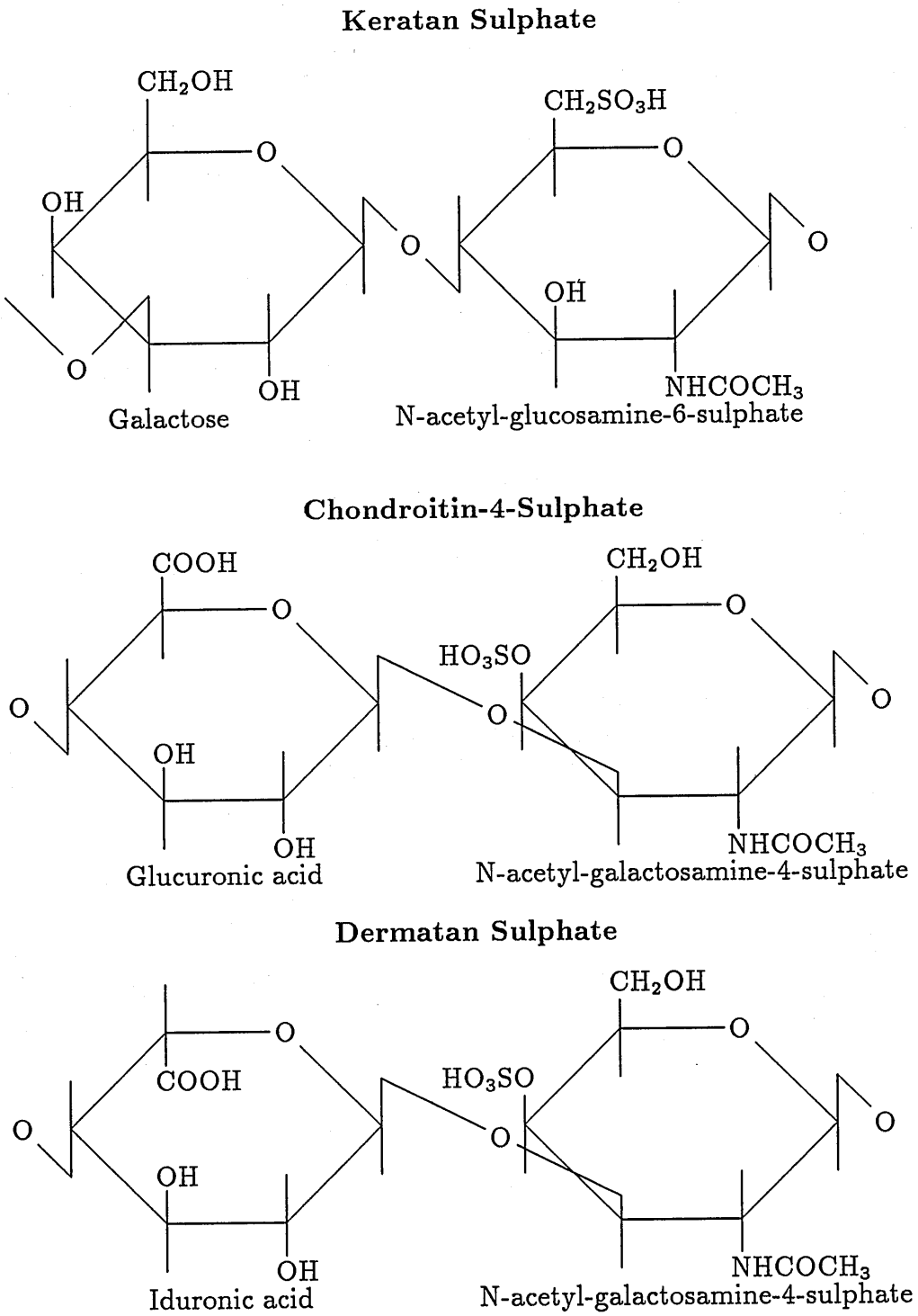


Figure 1.7 Chemical structures of keratan sulphate, chondroitin-4-sulphate and dermatan sulphate.

blue was altered by addition of magnesium chloride, confirmed the above assignments and further showed that the populations of KSPG at the a and c bands differed [Scott and Haigh, 1988].

Assignment of two different types of KSPG, namely PKSI(PKSA) and PKSII(PKSB), to the c and a bands of the collagen fibril respectively was originally proposed on the basis of the correlation between tissue concentration of the two types and frequency of occupancy of the c and a bands [Haigh and Scott, 1986]; similarly PDSI and PDSII were assigned to bands d and e respectively [Scott and Haigh, 1985].

While corneas from different animals may contain similar populations of proteoglycans , their location with respect to the staining bands of the collagen molecule may not be the same.

1.3.7 Other proteins found in cornea

BCP 54 (Bovine corneal protein)

This is the major soluble protein of bovine cornea, comprising approximately 30% of the total soluble protein [Alexander *et al.*, 1981]. Immunological studies of its tissue and species specificity indicate that it is present in the epithelium, stroma and endothelium of bovine cornea, and also bovine lens epithelium, but not in other tissues of the eye: proteins immunologically related to BCP 54 are present in other mammalian corneas, but studies to date have not shown its presence in non-mammalian corneas [Silverman *et al.*, 1981].

BCP 54 is composed of a single polypeptide chain of M_r 54 000, with a degree of microheterogeneity as shown by the presence of several close bands on isoelectric focussing. Following polyacrylamide gel electrophoresis, it does not stain with periodic acid-Schiffs reagent, and thus does not contain attached neutral sugars [Alexander *et al.*, 1981]; it does not stain with Alcian blue when used under 'critical electrolyte concentration' conditions [Wall and Gyi, 1988], indicating that it does not contain glycosaminoglycans. The amino acid composition of purified BCP 54 is shown in table 1.3.

Amino acid	Residues/1000 residues	Amino acid	Residues/1000 residues
Asp	88.8	Met	ND‡
Thr	58.5	Ile	48.9
Ser	79.6	Leu	67.9
Glu	114.8	Tyr	34.7
Pro	67.4	Phe	31.5
Gly	88.6	Lys	52.0
Ala	75.3	His	26.2
Cys†	23.1	Arg	62.8
Val	80.1		

Table 1.3 Amino acid composition of BCP 54, taken from Alexander *et al.*, 1981. †Expressed as half cystine; determined as cysteic acid after formic acid oxidation. ‡Not determined.

As can be seen from table 1.3, BCP 54 has a greater content of acidic compared to basic amino acids. No hydroxyproline or hydroxylysine has been found in BCP 54, and it does not contain a particularly high proportion of glycine, thus it is not a collagenous protein. The absence of 3-methylhistidine indicates that it is not an actin-like protein. It is not known whether BCP 54 has any enzyme activity, so although its abundance in and restriction to corneal tissues would indicate an important corneal-specific function, possibly in the maintenance of structure or transparency, its role remains obscure. It has been suggested that BCP 54 may be a proteoglycan core protein.

Other soluble proteins

A variety of other soluble proteins have been extracted from corneal stroma, including serum proteins such as albumin, immunoglobins and transferrin [Holt and Kinoshita, 1973]. Non-serum proteins specific to the cornea are also present in the extracts.

Oxytalan fibres

These are a type of elastic fibre found in cornea. They are composed of bundles of fibrils, each of which are 10–14nm in diameter, the bundles occurring between the collagen fibrils. They are most apparent in young animals, the amount decreasing as the cornea matures, or in certain pathological states. Studies of developing cat cornea have found oxytalan fibres distributed throughout the cornea in the newborn animal, whereas in the adult animal they appear to be restricted to the corneal periphery [Carrington *et al.*, 1984]. In man oxytalan has not been found in adult cornea, but is present in the juvenile and also in certain disease states, where the fibres may progress to become elaunin or elastic fibres.

Ultrastructural analysis of oxytalan fibres in rabbit cornea has shown that they are resistant to hyaluronidase and chondroitinase ABC, and thus are unlikely to contain glycosaminoglycan. Treatment of the stroma with non-specific protease or elastase results in degradation of the fibre bundles, thus indicating that they are composed of protein and are related to elastic fibres [Carlson and Waring, 1988]. The fact that oxytalan fibres are present in growing animals and in certain pathological conditions suggests that they may be produced in response to altered patterns of stress within the cornea.

1.3.8 Glycoproteins of the cornea

Keratonectin

Robert and coworkers have studied a glycoprotein fraction extractable from cornea using denaturing agents such as 8M urea [Robert, 1986]. The monomeric M_r of the glycoprotein is 34 000, although it is thought that this may be a degradation product [Alper, 1983]. The glycoprotein appears to exist as a dimer in the intact tissue, and it contains 5–10% sugar. It is synthesised by keratocytes and exported to the extracellular matrix, where it becomes strongly associated with the collagen fibres. Developmental studies have shown that the glycoprotein described here is synthesised in place of fibronectin in the chick cornea once fibronectin production ceases,

hence it has been named keratonectin. The amino acid composition of the purified glycoprotein is shown in table 1.4.

Amino acid	Residues/1000 residues	Amino acid	Residues/1000 residues
Allo Hylys	3.8	Gly	105.3
Hylys	7.6	Ala	70.1
Lys	44.2	Val	86.3
His	15.9	Cys	15.2
Arg	59.8	Met	10.8
Hypro	-	Ile	46.9
Asp	102.1	Leu	96.5
Thr	50.9	Tyr	17.4
Ser	59.3	Phe	37.7
Pro	56.6	NH_4	94.5
Glu	113.2		

Table 1.4 Amino acid composition of corneal structural glycoprotein [Robert, 1986]. Allo Hylys is a stereoisomer of hydroxylysine.

One suggested role for structural glycoproteins is in the regulation of collagen fibril diameter. It has been shown that the rate of incorporation of labelled amino acids into structural glycoproteins varies inversely with collagen fibre diameter [Robert and Parlebas, 1965]. Abnormal collagen fibre distributions in diseases such as keratoconus are accompanied by modified patterns of amino acid incorporation into structural glycoproteins in the stroma [Junqua *et al.*, 1975].

Structural glycoprotein complex

The original protocol developed by Robert to remove glycoproteins from the corneal stroma was modified by Alper (1983) to reduce proteolysis of the glycoprotein complex. Again a large disulphide-linked complex was extracted, which upon polyacrylamide gel electrophoresis revealed glycoproteins of M_r 155 000, 137 000, 117 000, 82 000 and 34 000. The amino acid composition of these components is shown in table 1.5.

	155 000	137 000	117 000	82 000	34 000
Hypro	17.2	17.4	17.6	-	-
Asp	74.0	72.0	66.7	75.1	62.5
Thr	43.1	41.2	40.6	56.9	45.3
Ser	66.7	67.0	80.2	86.6	157.3
Glu	123.3	127.2	127.1	120.6	146.4
Pro	53.8	47.8	48.5	24.7	tr?
Gly	188.8	203.8	241.1	155.5	251.7
Ala	67.7	63.7	63.8	80.2	79.7
Cys†	3.8	3.7	9.1	4.3	tr
Val	68.4	68.1	44.9	68.5	65.8
Met	10.3	10.0	10.2	18.3	7.1
Ile	37.0	38.2	33.8	45.8	30.7
Leu	68.5	68.3	62.7	108.7	66.8
Tyr	15.8	16.4	14.5	17.3	13.6
Phe	35.4	32.6	28.5	20.3	15.2
Hylys	15.8	14.1	14.1	-	-
Lys	32.6	36.7	30.5	46.1	tr
His	13.0	11.8	12.6	23.0	18.5
Arg	64.8	60.0	53.4	48.1	39.3

Table 1.5 Amino acid composition of corneal structural glycoprotein components [Alper, 1983]. †Expressed as half cystine.

The close association of this glycoprotein with the collagen fibres suggests a role in the structural organisation of the fibres; this interaction may be of vital importance in corneal development and repair. Further work has shown a relationship between the structural glycoprotein complex and type VI collagen [Alper and Amenta, 1986]; antibodies raised to both proteins produced virtually identical staining in the stroma of rat cornea.

Fibronectin

Fibronectin is a multifunctional glycoprotein which is involved in a range of diverse phenomena such as cell migration and adhesion, blood clotting and wound healing. It is present *in vivo* in both soluble and insoluble forms, the insoluble form being present in connective tissues. The soluble form is composed of two disulphide- bonded polypeptides (α and β chains) of M_r 220 000, which are immunologically very similar but which have different electrophoretic mobility on polyacrylamide gels. The insoluble form of fibronectin is poorly characterised.

In cornea, and also many other tissues, fibronectin is found in abundance during development, the amount decreasing as the tissue matures, indicating that fibronectin may function as an organiser in the formation of the corneal stroma. This would be reasonable since fibronectin contains binding sites for collagen, the presence of proteoglycans enhances the rate of binding of fibronectin to collagen, and stabilises the fibronectin-collagen complex.

Fibronectin also has a function in corneal wound healing. Following epithelial abrasion, fibronectin can be detected within hours in the anterior margin of the denuded stroma, where it is suggested that it provides a matrix for epithelial cell migration during wound closure [Tervo *et al.*, 1986].

Laminin and Nidogen

These are typical basement membrane components. In the mouse cornea they have been located in Descemet's membrane, the basement membrane of the corneal epithelium, and in a few basement membrane-like plaques

within the corneal stroma [Schittny *et al.*, 1988].

1.4 Corneal transparency

Transparency is an unusual property for a connective tissue. In order to attain this property, several factors are thought to be of importance in the cornea:

1. Uniform diameter of the collagen fibrils.
   Within corneas of individual species, collagen fibrils are narrow and uniform in diameter. Electron microscopic investigations by Craig and Parry (1981) originally gave fibril diameters of $\sim$ 24nm for a range of mammalian corneas, these were later modified to $\sim$ 35 – –39nm [Craig *et al.*, 1987] when using a low-temperature embedding technique which does not produce the shrinkage associated with conventional techniques. The larger diameters are in good agreement with those obtained by X-ray diffraction of wet, unfixed tissue [Sayers *et al.*, 1982,Worthington and Inouye, 1985]. When other non-transparent connective tissues are examined, there is found to be a vast range of diameters present e.g. tendon 30–300nm, human sclera 50– 160nm. It is interesting to note that in early stages of development the cornea and sclera are equally translucent, and at this stage their fibrils have equal diameters [Smelser, 1960].

2. Uniform spacing of the collagen fibrils.
   Maurice (1957) has suggested that the ordered arrangement of collagen fibrils which is found in cornea is an important factor in its transparency. In this lattice arrangement the fibrils act as a diffraction grating with a spacing which is less than the wavelength of light. Under these circumstances all scattered radiation is suppressed by destructive interference, leaving only the zero-order image unchanged, thus the cornea appears transparent. Loss of corneal transparency on swelling is explained very simply as a result of disordering of the fibril arrangement. The exact degree of regularity necessary to achieve

transparency has been considered by Hart and Farrell (1971), who have shown mathematically that transparency is possible with only short-range order of the collagen fibrils in the cornea. Benedek (1971) found that swollen corneas contain areas devoid of collagen fibrils called 'lakes', the presence of which causes increased light scattering resulting in loss of transparency.

3. Uniform refractive index.
   Prior to electron micrographs showing the corneal ultrastructure, transparency of the cornea was thought to be due to uniformity of refractive index within the cornea. However, the difference in density between the collagen fibrils and their surrounding ground substance is such that this theory of transparency is unlikely.

1.5 Objectives of this work

It is thought that the structural organization of the cornea, in particular the uniform size and spacing of its constituent collagen fibrils, is a major factor responsible for its transparency. The role of non-collagenous constituents in the maintenance of structural order in the cornea is not currently well understood, although proteoglycans and the glycosylation of collagen may be involved. *In vitro* systems show that proteoglycans are important in controlling collagen fibril diameter [Birk and Lande, 1981]. *In vivo* studies show that the rate of incorporation of labelled amino acids into structural glycoproteins in the cornea varies inversely with collagen fibril diameter [Robert and Parlebas, 1965], also, the degree of glycosylation of the collagen itself is related to the maximum diameter of fibrils found in different tissues [Harding *et al.*, 1980]. To understand fully the way in which the structure of cornea relates to its transparency, it is first necessary to characterise the components of the tissue, and to understand their spatial relationship to each other. With this in mind, the objectives of this work are:

1. to remove components from bovine, normal human and diseased human (keratoconus) corneas, using mild and harsh extraction procedures.

2. to analyse the components which have been removed, with respect to their molecular size and the presence of conjugated substances, namely neutral sugars and glycosaminoglycans.

3. to examine corneas after extraction in order to assess any alteration in ultrastructure and physical properties.

4. to try to correlate the removal of particular components with change in corneal structure, so as to assign a role for the components in the cornea.

1.5.1 Summary of methods used

To achieve the first objective, it was envisaged that mild extraction procedures using physiological salt solutions should remove soluble proteins from the cornea, whereas addition of chaotropic agents such as SDS and guanidinium chloride to the extraction solutions should remove components which are more strongly bound to the collagen framework.

For the second objective techniques such as polyacrylamide gel electrophoresis and differential gel staining were used. These techniques allow characterisation of extracted protein components with respect to their molecular weight and the presence of conjugated substances such as neutral sugars and glycosaminoglycans. Additional biochemical techniques were used to further characterise individual components.

To achieve the third objective, electron microscopic and X-ray diffraction analyses of corneas, processed as described above, were compared with analyses from untreated tissue. Electron microscopy provided information about the gross structural changes which occurred; while higher magnification electron microscopy and X-ray diffraction provided more detail relating to the structure of the collagen fibrils themselves. Comparison of

the electron density profiles of untreated and processed corneas showed where material had been removed from the collagen fibrils of the processed corneas.

It was hoped to be able to assign responsibility for changes in the X-ray pattern to particular extracted components. Processed corneas were examined using electron microscopic techniques in order to examine the gross structural effects of the extraction treatment, and changes in collagen fibril staining. Finally, the effect of extraction on transparency of the corneas was considered.

Chapter 2

Materials and Methods

2.1 Sources of chemicals and other materials

In general, chemicals used were of analytical reagent grade and were either obtained from Sigma Chemical Co. Ltd., Dorset, U.K. or BDH Ltd., Warwicks., U.K.. Other sources, or sources of important chemicals, are listed below.

Electrophoresis	
Acrylamide	National Diagnostics, Bucks., U.K. or BDH Ltd., Warwicks., U.K.
N,N',methylenebisacrylamide	National Diagnostics, Bucks., U.K. or BDH Ltd., Warwicks., U.K.
TEMED	Sigma Chemical Co. Ltd., Dorset, U.K.
Ammonium persulphate	BDH Ltd., Warwicks., U.K.
βmercaptoethanol	BDH Ltd., Warwicks., U.K.
SDS	Sigma Chemical Co. Ltd., Dorset, U.K.
Tris buffer	Sigma Chemical Co. Ltd., Dorset, U.K.
Molecular weight markers	Sigma Chemical Co. Ltd., Dorset, U.K.
Coomassie blue	Sigma Chemical Co. Ltd., Dorset, U.K.
Alcian blue 8GX	Sigma Chemical Co. Ltd., Dorset, U.K.
Schiffs reagent	BDH Ltd., Warwicks., U.K.

Electroblotting

Colloidal gold†	Janssen pharmaceuticals Ltd., Oxon, U.K.
Nitrocellulose paper	LKB/Pharmacia Bucks., U.K.

Electron microscopy

Glutaraldehyde	Agar aids, Essex U.K.
Epon resin	Taab 812 resin kit, Berks., U.K.
Spurr resin	Taab Spurr resin kit, Berks., U.K.
Lowicryl resin	Lowicryl K4M, Agar aids, Essex, U.K.
Uranyl acetate	BDH Ltd., Warwicks., U.K.
Colloidal gold†	Biocell Research Labs., S. Glamorgan, U.K.
Ilford electron microscope film	Ilford, Cheshire, U.K.

†In both cases these were goat anti rabbit secondary antibodies linked to 5nm gold particles.

2.2 Preparation of corneas

2.2.1 Bovine corneas

Bovine eyes were obtained fresh from the local abattoir, the corneas were excised, then epithelial and endothelial cell layers were removed by gentle scraping with a scalpel blade. The corneas were wrapped in cling film to preserve normal hydration and stored at $-20°C$ until used.

2.2.2 Human corneas

Normal and diseased human corneas were obtained as soon as possible post mortem, or post operatively in the case of specimens which had been removed prior to grafting. The corneas were wrapped in cling film and stored at $-20°C$ as for bovine corneas. The epithelial and endothelial cell layers were removed by scraping prior to use. Human corneas were always obtained with the cooperation of our medical colleagues, and under the general supervision of our clinical consultants (Trudi Blamires FRCS and

more recently Professor A.J. Bron FRCS).

2.2.3 Extraction procedures

Corneas were subjected to a variety of extraction procedures in order to compare proteins obtained under different conditions, and to see the effect of extraction on the corneas.

Extraction procedures including reducing agent

After thawing, corneas were extracted by gentle stirring in 0.15M NaCl plus 5%(w/v) βmercaptoethanol for 48h at 25°C, using 25ml extracting solution/g wet weight of tissue. Individual corneas were then bisected (across a diameter) and one half was retained in the salt/reducing agent solution. The remaining half was transferred to 0.15M NaCl plus 5%(w/v) βmercaptoethanol plus 1% SDS. The two groups of corneal sections were stirred for a further 24h after which samples of the extracts were retained and stored at -20°C. The two groups of corneal sections were then washed in 0.15M NaCl for a further 72h at 4°C prior to being dried down to approximately physiological hydration (see drying method below). The corneal sections were then stored at - 20°C until analysis by X-ray diffraction or electron microscopy.

Extraction procedures without reducing agent

After thawing, corneas were extracted by gentle stirring in 0.15M NaCl for 72h at 4°C, using 10ml extracting solution/g wet weight of tissue. The corneas were then divided into two groups, one group consisted of corneas extracted in fresh 0.15M NaCl at 4°C for 1,3 or 5 days the other group consisted of corneas extracted in 0.15M NaCl plus 1% SDS at 4°C for 1,3 or 5 days. Following extraction, all corneas were washed in fresh 0.15M NaCl for 48h at 4°C, prior to being dried down to approximately physiological hydration (see drying method below). There was a minor problem with this protocol in that the SDS tended to precipitate at low temperatures,

however this did not appear to affect its extraction efficiency.

A second protocol was also used, which was exactly the same as the one just described, but replacing 1% SDS with 4M guanidinium chloride.

The following protease inhibitors were included in all extraction solutions, 0.01% trypsin inhibitor, 2.5mM benzamidine hydrochloride, and 5mM disodium EDTA.

2.2.4 Corneal drying method

Swollen, extracted corneas were dried to approximately physiological hydration by placing them in short lengths of dialysis tubing with clips on either side, then immersing these in either 20% polyethylene glycol plus 0.15M NaCl at 4°C until the correct hydration was reached, or in 5% polyethylene glycol plus 0.15M NaCl until equilibrium, which produced the correct hydration. The corneas were put into dialysis tubing to prevent direct contact with the drying solution, so that the polyethylene glycol could not diffuse into the corneas or adhere to their surfaces. This method of drying corneas is more suitable than air drying as it allows even distribution of water throughout the cornea, whereas with air drying there is the problem of surface drying of the cornea while the interior remains hydrated. It also allows more precise control of hydration than air drying.

2.3 Electrophoresis

2.3.1 Polyacrylamide gel electrophoresis

For separation of extracted corneal components, SDS- discontinuous polyacrylamide gel electrophoresis was performed according to Laemmli (1970). Final concentrations of resolving gel chemicals were:
0.375M Tris-HCl pH8.8
0.1%(w/v) SDS
0.075%(w/v) ammonium persulphate
0.03%(v/v) TEMED

and generally
7.5%(w/v) acrylamide
0.2%(w/v) N,N',methylenebisacrylamide
where these vary it is noted.

Final concentrations of stacking gel chemicals were:
0.125M Tris-HCl pH6.8
0.1%(w/v) SDS
0.075% ammonium persulphate
0.05%(v/v) TEMED
3.75%(w/v) acrylamide
0.1%(w/v) N,N',methylenebisacrylamide.

Reservoir buffer was composed of 0.025M Tris, 0.192M glycine pH8.3.

The apparatus used for running the gels was an LKB 2001 vertical slab gel electrophoresis system as shown in figure 2.1. Samples prepared as described below were loaded into preformed sample wells in the stacking gel. Sample volumes varied according to the amount of protein present but were generally not greater than 150μl. Gels were generally run in pairs using an applied voltage of 150V with a current of approximately 110mA; under these conditions total run time was in the region of 5 hours.

2.3.2 Sample preparation for PAGE

Samples for electrophoresis were prepared by adding equal volumes of extract to 2X concentrated sample buffer, which contained 0.12M Tris-HCl pH 6.8, 20%(v/v) glycerol, 4%(w/v) SDS, 10%(v/v) βmercaptoethanol and 0.002%(w/v) bromophenol blue. The prepared samples were heated at 100°C for 2 minutes to dissociate completely the proteins present.

Molecular weight markers

Two different sets of molecular weight markers were used containing the following proteins

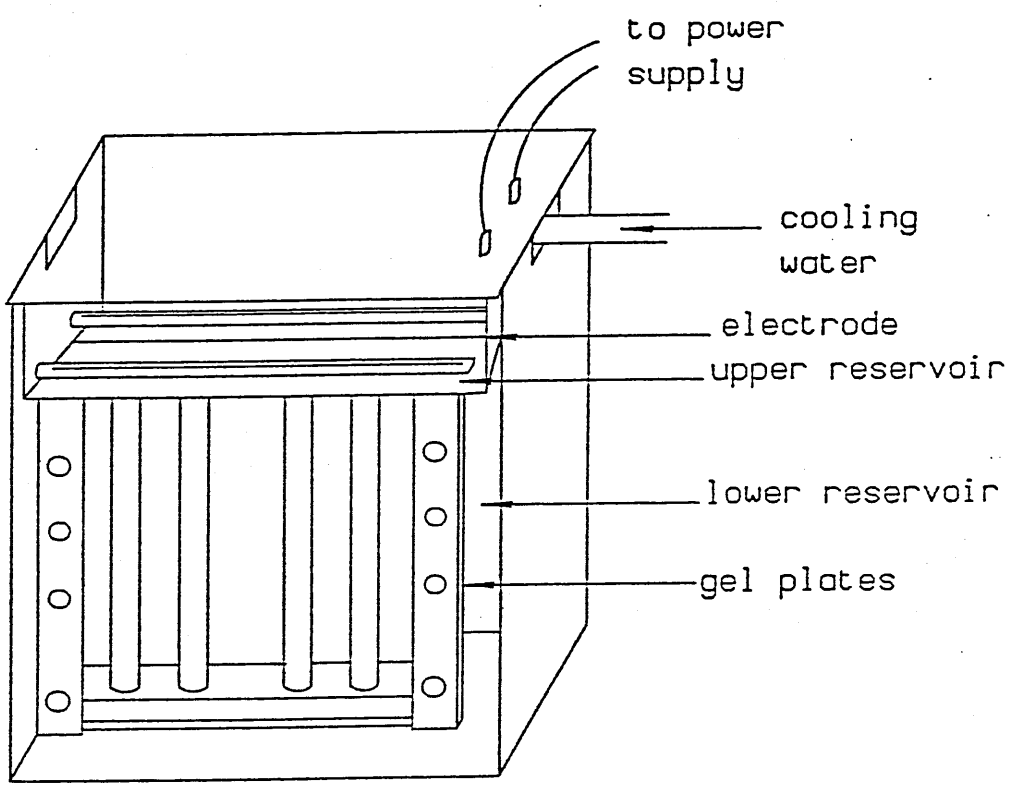


Figure 2.1 Diagrammatic representation of LKB 2001 vertical gel electrophoresis system.
Diagram courtesy of Dr. T.J. Gyi.

High MW markers	M_r
Myosin	205 000
βGalactosidase	116 000
Phosphorylase b	97 400
Bovine albumin	66 000
Egg albumin	45 000
Carbonic anhydrase	29 000

Prestained markers	M_r
α_2Macroglobulin	180 000
βGalactosidase	116 000
Fructose-6-phosphate kinase	84 000
Pyruvate kinase	58 000
Fumarase	48 500
Lactic dehydrogenase	36 500
Triosephosphate isomerase	26 600

2.3.3 Molecular weight determination in polyacrylamide gels

The migration of proteins in a polyacrylamide gel system depends on their molecular size, shape and charge. In systems which employ a non-dissociating buffer, proteins remain in their native conformation, and all three of the above characteristics contribute to their migration. In systems using a dissociating buffer, notably those which include the negatively charged ionic detergent SDS, proteins are no longer in their native conformation. A reducing agent such as βmercaptoethanol is also added to the buffer system, which breaks any inter- and intra-chain disulphide bonds and allows unfolding of the protein into an extended rod-like shape. SDS is bound to the protein in a constant weight ratio of 1.4g SDS per gram of protein [Hames and Rickwood, 1981]. Under these conditions, the charge attributable to the protein itself is insignificant compared to the negative charge provided by the bound SDS. Thus all proteins have similar hydrodynamic shape and charge densities, and can now be separated on the basis

of size alone. Comparison of the migration of sample proteins with those of known molecular weight standards allows determination of their molecular weight. This is done by plotting a graph of log (molecular weight) versus R_f for the standard proteins, and reading off the molecular weight of the sample proteins [Shapiro *et al.*, 1967].

Anomalous migration of some proteins can occur in this system. Where the charge on a protein is particularly high, this may alter its migration so that it runs slightly faster or slower than would be expected for its size. A different phenomenon occurs when collagens are separated using this system; they tend to migrate more slowly than globular proteins of comparable molecular weight. The hydrodynamic shape of collagens and globular proteins is supposedly similar after treatment with SDS, and they do not differ markedly in charge, so these cannot account for the altered migration. Furthmayr and Timpl (1971) propose that the difference is caused by variation in imino acid content, which alters the flexibility of the protein; those with high imino acid content such as collagens being less flexible than globular proteins with a lower content. The decrease in flexibility leads to retardation during molecular sieving, hence the slower migration of collagens on polyacrylamide gels. For this reason the molecular weights of collagenous proteins are sometimes quoted in the literature with reference to known collagenous standards: during the course of this work, molecular weights are quoted with reference to known globular protein standards.

2.3.4 Staining protocols for polyacrylamide gels

Coomassie blue protein stain

Following electrophoresis, gels were removed and fixed in 12.5% trichloroacetic acid overnight. They were then transferred to the staining solution, containing 1.25g Coomassie blue in 500mls of 10%(v/v) acetic acid/50%(v/v) methanol, until the gel had taken up stain completely (approximately 6h). Following staining, gels were destained in several changes of 5%(v/v) methanol/7%(v/v) acetic acid, until the background was clear.

Periodic acid-Schiffs staining for neutral carbohydrate

The method used was adapted from that of Fairbanks *et al.* (1971). Following electrophoresis, gels were soaked in 50%(v/v) methanol/7%(v/v) acetic acid for at least 1h. They were then transferred to 1%(w/v) periodic acid in 7%(v/v) acetic acid for 1h, during which time they were stored in the dark. Gels were then washed in several changes of 7%(v/v) acetic acid for at least 4h in order to remove excess periodate from the gel matrix. They were then transferred to Schiffs reagent for 1h in the dark, after which proteins containing attached neutral carbohydrate could be seen as pale pink bands against a clear background. Gels were then destained in several changes of 1%(w/v) sodium metabisulphite in 0.1M HCl, to remove excess Schiffs reagent from the background of the gel.

Toluidine blue staining for glycosaminoglycans

Following electrophoresis gels were fixed overnight in 12.5%(w/v) trichloroacetic acid, stained for 6h at room temperature in 0.1%(w/v) toluidine blue in 1%(v/v) acetic acid, then destained using several changes of 5%(v/v) methanol/7%(v/v) acetic acid.

Alcian blue staining for glycosaminoglycans

In the original method adopted, gels were first fixed overnight in 12.5%(w/v) trichloroacetic acid, stained using 0.5%(w/v) Alcian blue in 3%(v/v) acetic acid, then destained in several changes of 5%(v/v) methanol/7%(v/v) acetic acid. Subsequently, the method was adapted to include $MgCl_2$ in the stain and destain solutions, to increase the specificity of staining and reduce the level of background staining [Wall and Gyi, 1988]. Using this method, gels were fixed in 50%(v/v) methanol/7%(v/v) acetic acid for 1h, then washed in distilled water for 1h, then the two steps were repeated. These washings were found to be necessary in order to remove SDS thoroughly from gels prior to staining, otherwise extensive stain precipitation was observed. After washing, gels were stained overnight in 0.2% Alcian blue in 3%(v/v)

acetic acid solution containing 0.05M $MgCl_2$. Gels were then destained in several changes of 3%(v/v) acetic acid containing 0.05M $MgCl_2$, until the gel background was clear.

2.4 Other biochemical methods

2.4.1 Purification of GP135 by preparative gel electrophoresis

In the results section, an interesting glycoprotein of M_r 135 000 will be described, and is designated GP135. Small amounts of pure GP135 and BCP54 were required to perform amino acid analysis and for the raising of antibodies. These were prepared in the following way. SDS- PAGE of suitable corneal extracts was carried out on 7.5% polyacrylamide gels, a sample strip from the side of the gel was cut off and stained using Coomassie blue and destained in order to localise proteins in the gel, and the BCP54 or GP135 band cut out from the remaining gel. In early experiments the comb was not used during gel preparation so that the sample could be loaded across the entire stacking gel surface. In later runs the comb was included so that ten samples were run per gel. Generally the gel sections cut out for use were unfixed and unstained but in some cases the gels were stained lightly with Coomassie blue so that the relevant bands could be cut out more accurately.

2.4.2 Production of antisera to BCP54 and GP135

This work was done in collaboration with Dr. C.J. Branford-White at the University of Bath.

The method used for the raising of antibodies was based on that of Burrin, (1983). Small strips of polyacrylamide gel containing BCP54 or GP135 were cut out of gels run as described above and emulsified with Freund's incomplete adjuvent. This was used to inoculate rabbits subcutaneously, then a booster injection was given six weeks later. Samples of

blood from immunized rabbits were obtained one week after the booster injection, these were allowed to clot, then centrifuged to obtained cell-free serum. The serum was incubated at 56°C for 45min to inactivate proteases and complement.

2.4.3 Amino acid analysis

This work was done in collaboration with Dr. C.J. Branford-White at the University of Bath.

Following purification of GP135 by preparative gel electrophoresis, unfixed and unstained gel sections containing GP135 were cut out. The gel sections were then soaked for approximately 3 days in a small volume of 1% SDS in order to elute the protein from the gel section. The eluate was then dialysed extensively vs. distilled water to remove the SDS and any remaining glycine from the original gel running buffer. The sample was subsequently bubbled with nitrogen and hydrolysed in constant boiling 6M HCl under vacuum at 105°C for 24h. Amino acids in the hydrolysate were analysed using a Rank Hilger Cromaspek II.

2.4.4 Pepsin digestion of corneal extracts

Using the method of Ayad *et al.*, (1985) a guanidinium chloride extract from bovine cornea was dialysed vs distilled water and treated with pepsin to produce collagenous fragments. These were then analysed using polyacrylamide gel electrophoresis and compared with pepsinised type I collagen obtained from calf skin (Sigma Chemical Co., Poole, Dorset, U.K.)

2.4.5 Western blotting of corneal extracts

This method, used for the transfer of proteins in polyacrylamide gels onto nitrocellulose paper, was based on the procedure described by Towbin *et al.* (1979), using an LKB2117–250 Novablot electrophoretic transfer kit. Figure 2.2 shows a diagrammatic representation of the assembled apparatus. Prior to blotting, gels were soaked for 30–60 min in electroblotting buffer

solution containing:
39mM glycine
48mM Tris
0.0375%(w/v) SDS

For the blotting procedure, the graphite electrodes were soaked with distilled water, then blotted. Two stacks of nine filter papers and a sheet of nitrocellulose paper were cut to the same size as the gel to be blotted, wetted with electroblotting solution and then assembled in the order shown in figure 2.2. During the wetting and assembly process, care was taken not to trap air bubbles in the filter papers or between layers as this would have affected the current passed. Following assembly, a current of $0.8mA/cm^2$ gel area was applied for approximately 2h to transfer proteins onto the nitrocellulose. After transfer the nitrocellulose membrane was air dried, and was then ready to use in subsequent blotting or staining procedures.

2.4.6 Staining of nitrocellulose membrane for proteins

To check blotting efficiency, nitrocellulose strips corresponding to lanes of the original gel were stained for protein using the following method. Strips were washed for 10 min in washing solution containing:
17.5g NaCl
91mls 0.2M $Na_2HPO_4.12H_2O$
15mls 0.2M $NaH_2PO_4.2H_2O$
6mls Tween 20
The above made up to 1 litre with distilled water.

The washing step was repeated 3 times, then the strips were placed in staining solution, containing $500\mu l$ indian drawing ink (Pelican fount) in 500ml washing solution, for at least 2h. Strips were then washed for 2 min in distilled water, washed again in fresh distilled water and then dried.

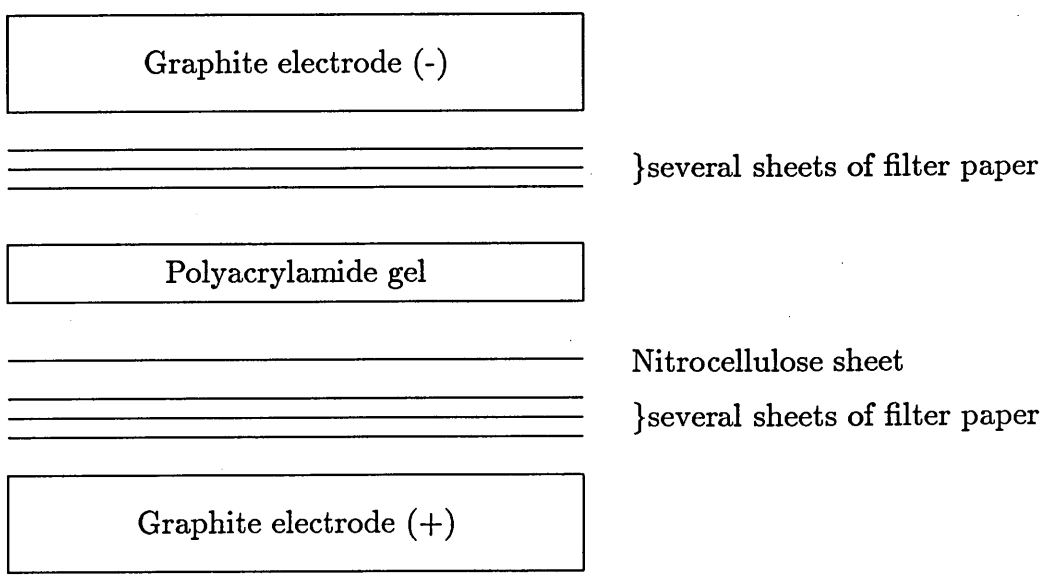


Figure 2.2 Diagram showing assembly of electroblotting apparatus

2.4.7 Immunoblotting procedure for specificity testing of raised antisera

The following solutions were freshly prepared for the immunoblotting procedure.

1. Washing solution
   0.1% BSA
   20mM Tris-HCl pH 8.2
   0.15M NaCl

2. Blocking solution
   5% BSA
   20mM Tris-HCl pH 8.2
   0.15M NaCl

3. Antibody solution
   Serum following immunization diluted in washing solution. Dilution depends on particular serum used.

4. Gelatin solution (supplied by Janssen)
   1 in 20 dilution of gelatin in washing solution. This is a specially prepared gelatin preparation supplied by the colloidal gold manufacturers. The gelatin was warmed to reduce its viscosity prior to pipetting.

5. Gold solution (supplied by Janssen)
   1 in 100 dilution of 5nm colloidal gold particles linked to goat anti rabbit secondary antibodies in gelatin solution.

6. Silver solution (supplied by Janssen)
   Equal volumes of enhancer and initiator solutions.

Blotting procedure

During this procedure the incubations were performed on a rocking table to agitate the nitrocellulose membrane in the various solutions. The mem-

brane was:

1. Placed in blocking solution at 37°C for 30 min. The remaining steps were performed at room temperature.
2. Washed 3 times for 5 min in washing solution.
3. Incubated in primary antibody for 1–2h.
4. Washed 3 times for 5 min in washing solution.
5. Incubated in gold solution for 2h.
6. Washed twice for 5 min in washing solution.
7. Washed twice for 1 min in distilled water.
8. Incubated in silver solution for 10–20 min.
9. Washed in several changes of excess distilled water.
10. Dried between filter paper.

2.5 Electron microscopy

2.5.1 Preparation of resins

The following proportions of components were used for making up resins.

Epon resin

Mix A	
Epon 812	5ml(6.40g)
DDSA	8ml(7.53g)
Mix B	
Epon 812	8ml(10.77g)
MNA	7ml(8.38g)

Mix A and Mix B added together, then 16 drops (0.19g) BDMA mixed in, mixture allowed to stand and degassed if necessary to remove bubbles.

Spurr resin

NSA	26g
ERL	10g
DER	7g
S1	0.6g

Above mixed well before use.

Lowicryl K4M resin

Crosslinker A	2.7g
Monomer B	17.3g
Initiator C	0.1g

Above mixed well before use.

Fixation procedure for Epon or Spurr resin embedding

The following fixation procedure was adopted for corneal tissue which was subsequently embedded in Epon or Spurr resin.

1. Samples were cut into small pieces (1–2mm) and fixed in 2.5% glutaraldehyde in 0.1M sodium phosphate buffer pH 7.0 for at least 2h.
2. Samples were washed twice for 30 min each in 0.1M sodium phosphate buffer pH 7.0.
3. Post-fixed for 1–2h in a 1:1 mixture of 4% osmium tetroxide, 0.2M collidine.
4. Washed thoroughly as in step 2.
5. Dehydrated in ethanol, 10–15min in each of 50%, 70%, 85%, 90%, 95% ethanol and finally twice in 100% ethanol for 15–20min.

Embedding procedure (Epon or Spurr resin)

6 Infiltrated for 10min in 1:1 propylene oxide and ethanol.

7 Washed twice in 100% propylene oxide for 15min each.

8 Infiltrated for 1.5h in 1:1 resin and propylene oxide.

9 Infiltrated for 3h in 3:1 resin and propylene oxide.

10 Infiltrated overnight in 100% resin.

11 Embedded in 100% resin, then baked for 24h at 60°C (Epon) or 24h at 40°C then 24h at 70°C (Spurr).

Fixation procedure for Lowicryl K4M resin embedding

The following procedure was adopted for corneal tissue which was subsequently embedded in Lowicryl K4M resin. This resin was chosen for immunostaining work as it is hydrophilic and preserves antigenic sites well compared with epoxy resins.

1. Samples were cut into small pieces (1–2mm) and lightly fixed in 0.5% glutaraldehyde in 0.1M sodium phosphate buffer pH 7.0 for 2–4h.

2. Samples were washed twice for 30 min each in 0.1M sodium phosphate buffer pH 7.0.

3. Samples were then dehydrated in ethanol using the progressive lowering of temperature technique, which helps to maintain the original tissue structure.

   (a) In 30% ethanol at 0°C for 30 min.

   (b) In 50% ethanol at −20°C for 60 min.

   (c) In 70% ethanol at −35°C for 60 min.

   (d) In 95% ethanol at −35°C for 60 min.

   (e) In 100% ethanol at −35°C for 60 min.

   (f) In 100% ethanol at −35°C for 60 min.

Embedding procedure (Lowicryl K4M resin)

4. Samples were then infiltrated for 60 min in 1:1 resin and ethanol at −35°C.

5. Infiltrated for 60 min in 2:1 resin and ethanol at −35°C.

6. Infiltrated for 60 min in pure resin at −35°C, then again at −35°C in pure resin overnight.

7. Gelatin capsules were filled with fresh pre-cooled resin, and samples transferred into these using pasteur pipettes.

8. Resin was polymerised for at least 24h under ultraviolet light at −35°C. The capsules were then removed from the cold and curing continued under ultraviolet light for 2–3 days at room temperature.

Subsequent processing of embedded material

Silver-gold (70nm) sections were cut from embedded material with a Dupont diamond knife using a Reichert-Jung Ultracut E ultramicrotome. The cut sections were relaxed with chloroform, then Epon and Spurr sections were picked up onto copper grids, or Lowicryl sections onto nickel grids. Positive staining using 0.5%(w/v) uranyl acetate in 50%(v/v) ethanol was performed by floating the grids face downwards onto the staining solution at 37°C for 30mins, then rinsing in 3 changes of 50% ethanol and drying on filter paper. Sections were examined using a Philips 301 transmission electron microscope running at 80kV. Photographic negatives were recorded using Ilford electron microscope film (Ilford, Cheshire, U.K.).

2.5.2 Immunogold staining procedure

Lowicryl K4M sections on nickel grids were used for this procedure. The grids were floated face down onto various solutions as detailed below.

1. Blocking solution; 1% BSA, for 10 min.

2. Primary antibody; dilutions of serum in PBS (phosphate-buffered saline) plus 0.1% BSA pH 7.4 for between 30 min and 18h.

3. Wash solution; grids were moved through 5 washes of 5 min each in PBS to remove unbound primary antibody.

4. Gold stain; grids were placed on colloidal gold solution pH 8.2 for 1h. This solution contained goat anti rabbit secondary antibodies linked to 5nm colloidal gold particles.

5. Wash; grids were moved through 5 washes of 2 min each in distilled water to remove unbound colloidal gold.

6. Stain; grids were stained using 5% uranyl acetate for 15 min.

7. Wash; grids were washed in distilled water to remove excess stain.

Grids were then stained positively using uranyl acetate as described in the previous section.

2.5.3 Negative staining and computer processing of isolated collagen

Small pieces of extracted corneas were ground in a few drops of distilled water with a mortar and pestle to produce a fine suspension. After several minutes of this treatment, a drop of the suspension was transferred to a plastic-coated copper support grid. The material was then negatively-stained by adding a drop of 1% phosphotungstic acid raised to pH 7.0 by addition of NaOH to the grid, then blotting it off. The grids were then examined using a Philips 301 transmission electron microscope running at 80kV. Photographic negatives were recorded using Ilford electron microscope film (Ilford, Cheshire, U.K.).

The photographic negatives were then scanned using a Joyce Loebl microdensitometer, along the axis of the collagen fibrils. Scans were then averaged from 20 different D periods to produce an average optical density profile.

2.6 X-ray diffraction

X-ray diffraction patterns were obtained at the high intensity synchrotron source at Daresbury, U.K.. Corneas were weighed, then mounted in airtight

perspex cells to keep the corneal hydration as constant as possible for the duration of the X-ray exposure. The cells contained mylar windows on both sides of the cornea to allow passage of X-rays. After exposure corneas were weighed again to ensure that their hydration had not changed significantly. The dimensions of the beam were generally 0.5mm(height), 4mm(width). The camera length used was between 2 and 3 metres, it was not necessary to measure the exact length of the camera, as a calibration pattern was recorded for each set of exposures. Rat tail tendon collagen was used for calibration, where the distance between the film centre and the first order reflection of the meridional pattern corresponds to a spacing of 67nm. X-ray patterns were recorded on either Kodak DEF 59 or Ceaverken CEA reflex 25 film. Exposure times using synchrotron X-rays are very short compared with those needed using conventional X-ray sets, where exposures in the region of 24h are necessary for corneal collagen. Exposures of approximately 15s were used to record the first order of the meridional pattern, 40mins to record up to the tenth order, and 5–10mins to record intermediate orders. It is not possible to record all the orders necessary for further analysis on one film, because diffuse scatter in the centre of the pattern quickly obscures the lower orders, hence the necessity to obtain a range of exposures. The wavelength of the radiation used was 0.154nm.

2.6.1 Analysis of X-ray patterns

X-ray patterns obtained as described above were scanned using an LKB 2222–010 Ultroscan XL laser densitometer to record the intensities of the meridional reflections. The integrated intensities for each reflection were measured by cutting out the area under each peak and weighing. Each of these integrated intensities was then multiplied by the order of the reflection to compensate for the fact that the densitometer made linear scans across a circular diffraction pattern. This produced a series of relative intensities, which could then be used to compute the axial electron density along the collagen fibril [Meek *et al.*, 1981a].

2.7 Transparency measurements

2.7.1 Preparation of corneas

For this experiment, 21 bovine corneas were extracted for 3 days initially in 0.15M NaCl, then 3 of these were removed for drying, and the remaining 18 split into 2 groups of 9. One group continued to be extracted in 0.15M NaCl for 1 day, the other group was transferred to 0.15M NaCl plus 4M guanidinium chloride. From each group, 3 corneas were washed in 0.15M NaCl (4 $\times$ 1 day), then dried; the remaining 6 continued to be extracted for an additional 2 days. Three of these were washed and dried, and the last 3 in each group continued to be extracted for an additional 2 days. These last 3 were then washed and dried.

Absorbance measurements for the corneas were obtained when the corneas were fresh, after the initial 3 day salt extraction, and after a further 1,3 or 5 days in either 0.15M NaCl or 0.15M NaCl plus 4M guanidinium chloride. Absorbance measurements were also obtained after the swollen corneas had been washed and dried down to approximately physiological hydration.

2.7.2 Measurement of corneal absorbance

Assessment of corneal transparency was made by scanning individual corneas using an LKB 2222–010 Ultroscan XL laser densitometer. The wavelength of light produced by the laser was 633nm. The shape of the beam was rectangular, of size 50μm by 800μm. When scanning transparent material the densitometer records an absorbance of zero. As cornea loses its transparency, the densitometer records increasing absorbances, as the light scattered by the cornea is no longer detected by the photocell in the machine. Increasing absorbance therefore relates to decreasing transparency in the cornea.

In order to reduce noise caused by the irregular surface of the cornea, a glass slide was placed on top of corneas being scanned so that its surface was smooth.

Chapter 3

Results

In this chapter, results will be presented from four main areas of study

1. Using biochemical methods to analyse components extracted from corneas: polyacrylamide gel electrophoresis using differential staining is the main method used. A detailed analysis of the components extractable under different conditions is presented. Following this work, further biochemical analyses are shown of important individual components identified on the original gels, most notably a glycoprotein with M_r 135 000 which is designated GP135. To continue investigation of GP135, antiserum was raised to this glycoprotein, and tested by immunoblotting of western blots of corneal extract, with the aim of using the antibodies subsequently in electron microscopical work to locate GP135 in the original structure of the cornea. Antiserum to BCP54 was also raised for the same reason.

2. Electron microscopical results are presented which show the change in structure of the cornea following extraction using mild and harsh methods. Preliminary results of immunoelectron microscopy using antibodies to GP135 are also shown.

3. X-ray diffraction results are presented which illustrate the alteration in structure of the cornea following mild and harsh extraction procedures.

4. Transparency measurements from extracted corneas are presented, which illustrate the difference in transparency of corneas extracted under mild and harsh extraction conditions.

3.1 Polyacrylamide gel electrophoresis of corneal extracts

In this section, polyacrylamide gels will be presented which show the components extractable from bovine cornea, and normal and keratoconus human corneas, under mild and harsh conditions of extraction. These gels have been stained in different ways to illustrate the chemical nature of the components extracted, ie whether they are protein alone, or protein conjugated with neutral sugar or glycosaminoglycan. Coomassie blue staining has been used to stain gels for protein, periodic acid/Schiffs reagent to stain for neutral sugar, and Alcian blue to stain for glycosaminoglycan.

3.1.1 Coomassie blue staining of bovine corneal extracts separated on polyacrylamide gels

Corneas extracted using NaCl, βmercaptoethanol and SDS

Figure 3.1 compares the pattern of proteins obtained when corneas are extracted using NaCl alone, NaCl plus βmercaptoethanol, and NaCl plus βmercaptoethanol plus SDS. Lane 1 contains molecular weight markers. Lane 2 shows the typical pattern of proteins obtained using NaCl alone. The molecular weights of the most prominent bands are shown; these are 54 000, 66 000, 45 000, 75 000 and a broad band of staining at M_r 180 000–200 000. Lane 3 shows the pattern when 5% βmercaptoethanol is added to the extracting solution, the staining pattern is virtually identical, with the addition of two proteins running slightly ahead of the M_r 66 000 band. Lane 4 shows the pattern obtained when 5% βmercaptoethanol plus 1% SDS is added to the extracting solution; it should be noted that this ex-

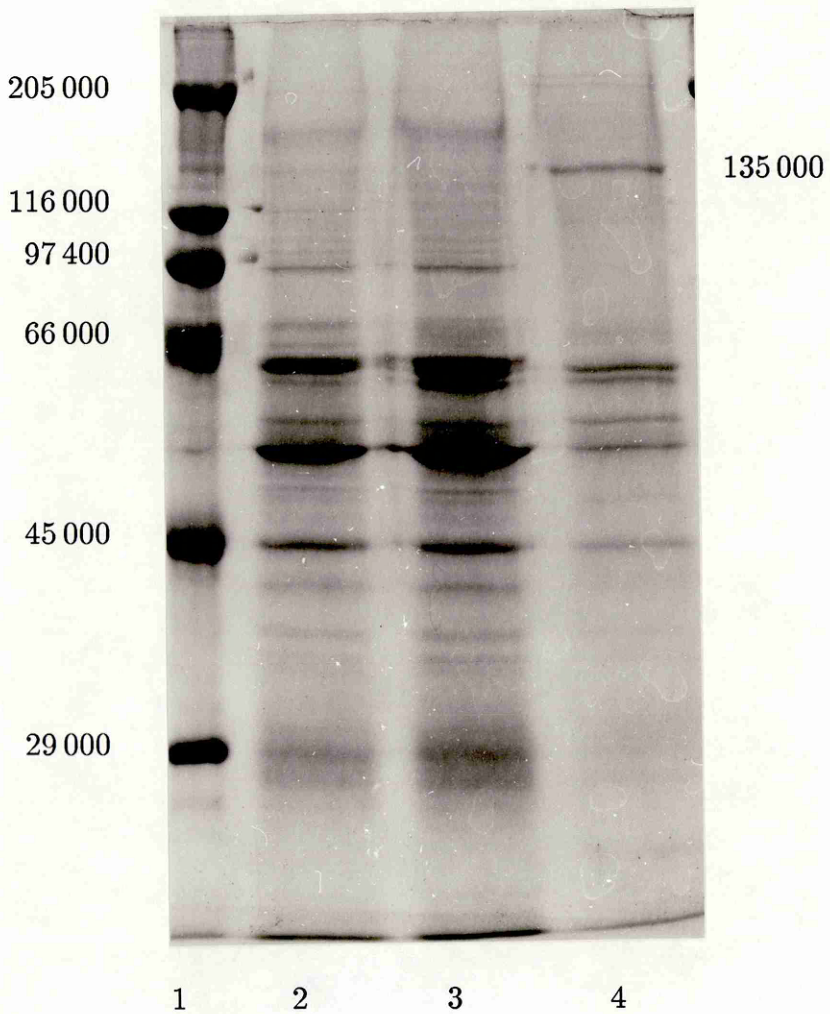


Figure 3.1 Coomassie blue staining of bovine corneal extracts following SDS-PAGE on 7.5% gel.
Lane 1: Molecular weight markers, M_r 205 000, 116 000, 97 400, 66 000, 45 000 and 29 000.
Lane 2: 0.15M NaCl extract.
Lane 3: 0.15M NaCl plus 5% βmercaptoethanol extract.
Lane 4: 0.15M NaCl plus 5% βmercaptoethanol plus 1% SDS extract.

traction is sequential to that of NaCl plus βmercaptoethanol, hence the reduction in amount of staining of the various bands. In this case it can be seen that most of the proteins which were obtained using NaCl plus βmercaptoethanol continue to be extracted, and there is an additional, prominent band of M_r 135 000 which was not present either in the NaCl or NaCl plus βmercaptoethanol extracts. This protein was subsequently found to stain using periodic acid/Schiffs reagent (see figure 3.4 page 59), thus it is a glycoprotein and is designated GP135. There are also two bands of high M_r ($\sim$ 200 000) which again were not present in the other two extracts.

Omission of βmercaptoethanol from the extraction medium

Corneas were extracted using NaCl and SDS, in the presence and absence of βmercaptoethanol, to see the effect of the reducing agent on the general pattern of proteins extracted (figure 3.2), and in particular to see if there was any difference in the extraction of the M_r 135 000 protein. Lane 1 shows the extract where NaCl, βmercaptoethanol and SDS were used, lane 2 shows the NaCl plus SDS extract (no βmercaptoethanol), lane 3 contains molecular weight markers. The pattern of proteins seen in lanes 1 and 2 are essentially identical, thus it is not necessary to use reducing agent in the extraction medium to remove the M_r 135 000 protein from the corneal matrix.

Corneas extracted using NaCl and SDS

Figure 3.3 shows the proteins extracted after 1, 3 and 5 days in NaCl or NaCl plus SDS. Lane 1 contains molecular weight markers. Lanes 2, 4 and 6 show the proteins in the extracts obtained following 1, 3 and 5 days respectively in NaCl alone. Lanes 3, 5 and 7 show the extracts obtained following 1, 3 and 5 days respectively in NaCl plus SDS. These are sequential extractions, hence the reduction in amount of staining with time.

After 1 day (lanes 2 and 3) the pattern of proteins obtained is virtually identical, with the exception of the M_r 135 000 protein, which is present in

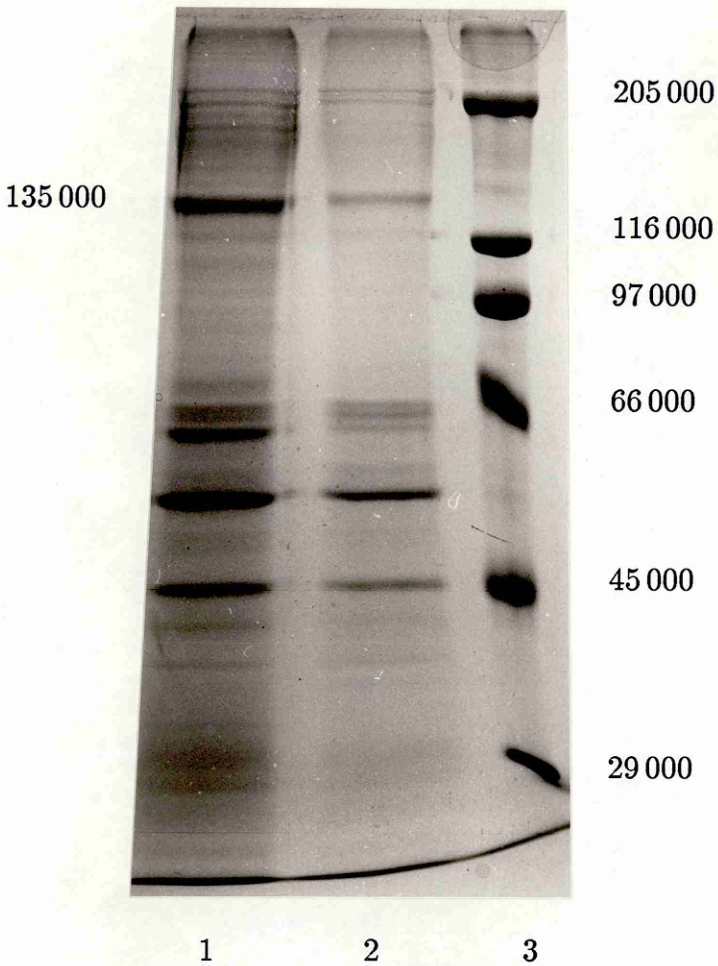


Figure 3.2 Coomassie blue staining of bovine corneal extracts used with and without reducing agent.
Lane 1: 0.15M NaCl plus 5% βmercaptoethanol plus 1% SDS extract.
Lane 2: 0.15M NaCl plus 1% SDS extract.
Lane 3: Molecular weight markers, M_r 205 000, 116 000, 97 400, 66 000, 45 000 and 29 000.

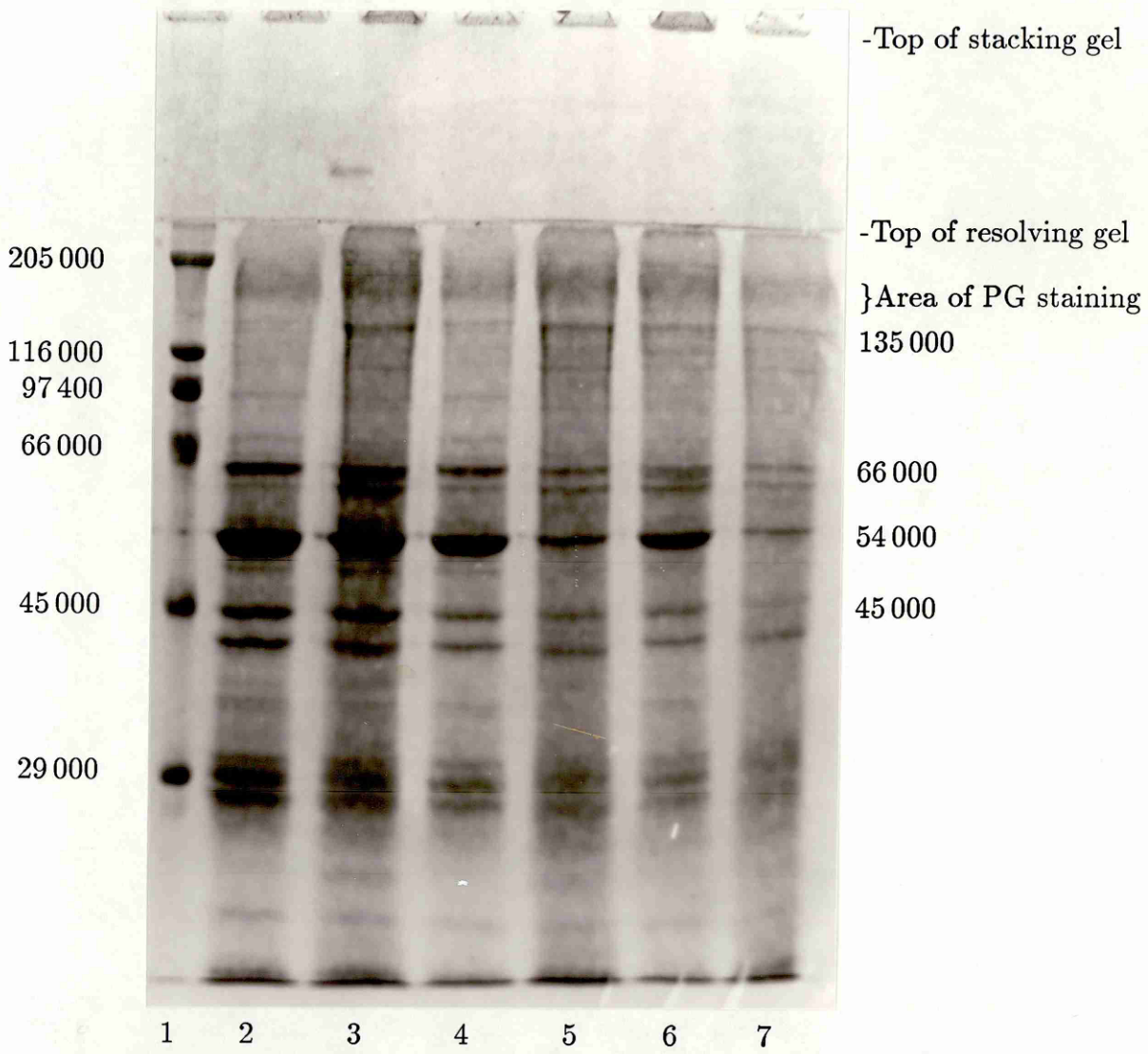


Figure 3.3 Coomassie blue staining of NaCl and SDS extracts.
Lane 1: Molecular weight markers, M_r 205 000, 116 000, 97 400, 66 000, 45 000 and 29 000.
Lane 2: 1 day 0.15M NaCl extract
Lane 3: 1 day 0.15M NaCl plus 1%SDS extract
Lane 4: 3 day 0.15M NaCl extract
Lane 5: 3 day 0.15M NaCl plus 1%SDS extract
Lane 6: 5 day 0.15M NaCl extract
Lane 7: 5 day 0.15M NaCl plus 1%SDS extract

the NaCl plus SDS extract but not in the NaCl only extract. Also there is a larger amount of the protein which runs ahead of the M_r 66 000 protein in lane 3, and a smaller amount of the M_r 54 000 protein. Similar results are seen after 3 days (lanes 4 and 5) with an overall reduction in staining due to lesser amounts of protein extracted. After 5 days, in the NaCl only extract, a new protein can be seen which has run slightly ahead of the M_r 66 000 protein, which is not present in the NaCl plus SDS extract. Thus the two proteins running ahead of the M_r 66 000 protein in lane 6 look very similar to those seen in the NaCl plus βmercaptoethanol extract of figure 3.1 lane 2. Lanes 6 and 7 contain comparable amounts of the M_r 135 000 protein. Lanes 2 to 7 all show in varying amounts the diffuse band of staining at M_r 180 000– 200 000.

3.1.2 Periodic acid-Schiffs staining of bovine corneal extracts separated on polyacrylamide gels

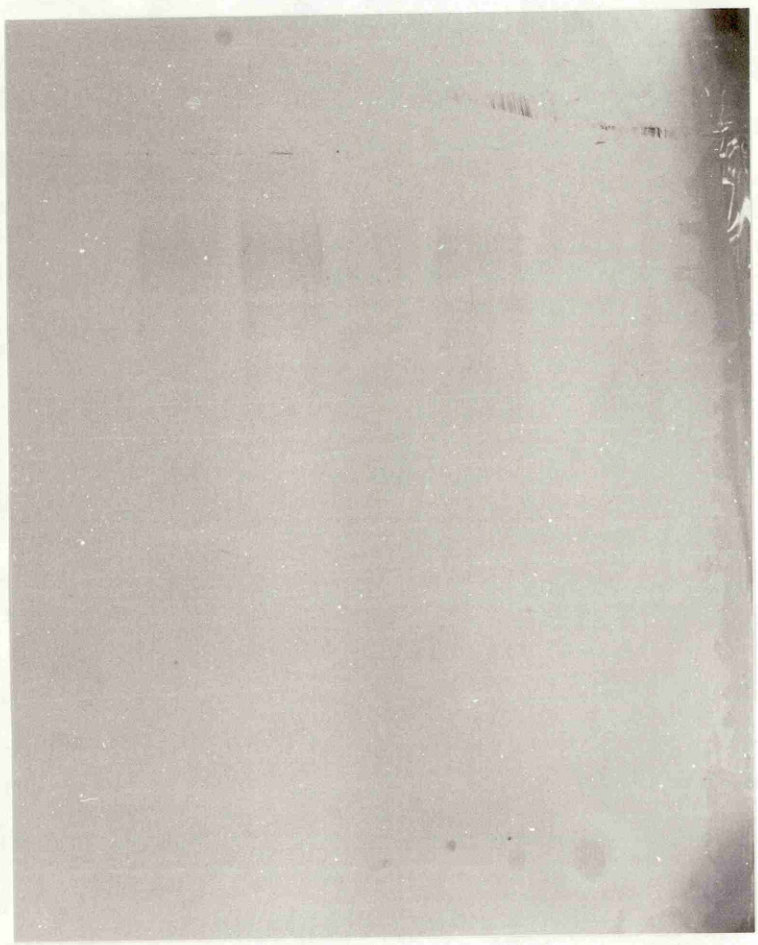
Corneas extracted using NaCl plus SDS

Figure 3.4 is the corresponding gel to that shown in figure 3.3, but stained for neutral sugar using periodic acid-Schiffs reagent. Staining is very faint, but it can be seen that there are two areas of staining in several of the lanes. One area corresponds to the M_r 135 000 protein where this is present, this can be seen most clearly in lanes 3 and 5, hence its designation GP135 as explained previously. The other area corresponds to the diffuse area of protein staining of M_r 180 000– 200 000, this can be seen in lanes 2 to 7. Lane 1 contains molecular weight markers, none of these have stained.

3.1.3 Alcian blue staining of bovine corneal extracts separated on polyacrylamide gels

Corneas extracted using NaCl plus SDS

Figure 3.5 is the corresponding gel to that shown in figure 3.3, stained for glycosaminoglycan with Alcian blue using the critical electrolyte concen-



Top of stacking gel

Top of resolving gel

}Area of PG staining
135 000

1 2 3 4 5 6 7

Figure 3.4 Periodic acid-Schiffs staining of NaCl and SDS extracts.
Lane 1: Molecular weight markers, M_r 205 000, 116 000, 97 400, 66 000, 45 000 and 29 000. These have not taken up stain, therefore molecular weights have been inferred from the corresponding protein stained gel, figure 3.3.
Lane 2: 1 day 0.15M NaCl extract
Lane 3: 1 day 0.15M NaCl plus 1%SDS extract
Lane 4: 3 day 0.15M NaCl extract
Lane 5: 3 day 0.15M NaCl plus 1%SDS extract
Lane 6: 5 day 0.15M NaCl extract
Lane 7: 5 day 0.15M NaCl plus 1%SDS extract

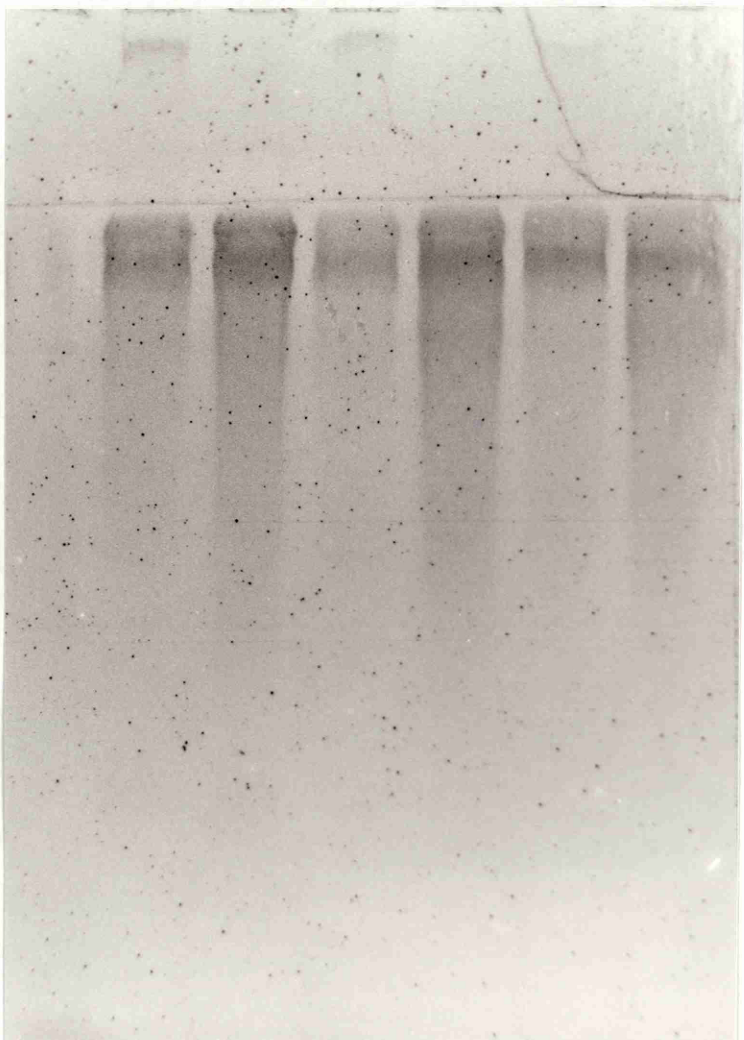


Figure 3.5 Alcian blue staining of NaCl and SDS extracts.
Lane 1: Molecular weight markers, M_r 205 000, 116 000, 97 400, 66 000, 45 000 and 29 000. These have not taken up stain, therefore molecular weights have been inferred from the corresponding protein stained gel, figure 3.3.
Lane 2: 1 day 0.15M NaCl extract
Lane 3: 1 day 0.15M NaCl plus 1%SDS extract
Lane 4: 3 day 0.15M NaCl extract
Lane 5: 3 day 0.15M NaCl plus 1%SDS extract
Lane 6: 5 day 0.15M NaCl extract
Lane 7: 5 day 0.15M NaCl plus 1%SDS extract

tration method [Wall and Gyi, 1988]. Lane 1 contains molecular weight markers, none of these have taken up the stain. Molecular weight ranges of the Alcian blue stained bands have been inferred from the corresponding Coomassie blue stained gel, figure 3.3.Two areas of staining can be seen in all the remaining lanes, with some variability in the amount of stain taken up. One area corresponds to the diffuse area of protein staining of M_r 180 000–200 000. The other area, running slightly behind the first, is again diffuse, covering a large molecular weight range, but it is not possible to estimate this accurately as it is above the position where the highest molecular weight marker would be if it were visible.

A third area of staining is present in the stacking gel, but only in lanes 2,4 and 6, which are lanes containing extracts where only NaCl was present for 1,3 and 5 days. Where SDS was present in the extracts the band is not apparent. This suggests selective removal of a very high molecular weight proteoglycan by NaCl only; the presence of SDS prevents the proteoglycan from being extracted. This band did not stain with coomassie blue on the corresponding protein gel (figure 3.3), or with PAS reagent on the corresponding gel stained for neutral sugar (figure 3.4).

Development of Alcian blue critical electrolyte concentration staining method for polyacrylamide gels

Figure 3.6 shows the results of Alcian blue staining following electrophoresis of corneal extracts. The staining method used was the original method as described in section 2.3.4. This gel is included for comparison with gels where the CEC method of staining was used, in order to demonstrate the improvement in clarity of the results obtained when using the new method.

Figure 3.7 shows the results of Alcian blue staining following electrophoresis of corneal extracts where different amounts of $MgCl_2$ were added to the stain and destain solutions. The corneal extract used in this case was a NaCl only extract. In lanes 1 and 2 no $MgCl_2$ was added; two broad areas of staining can be seen towards the top of the gel, plus a sharper band corresponding to a M_r of 54 000. Background staining in this case was fairly high. In lanes 3 and 4, 0.2M $MgCl_2$ was added to the stain and destain solutions; two broad areas of staining occur as before, but the M_r 54 000 protein no longer takes up stain. The background staining is very much reduced compared with the no $MgCl_2$ lanes. In lanes 5 and 6, 0.8M $MgCl_2$ was added to the stain and destain solutions, the staining pattern produced looks very similar to that of lanes 3 and 4. As in lanes 2,4 and 6 of figure 3.5, very high molecular weight proteoglycans can be seen in all lanes in the stacking gel. The intensity of staining of this band decreases with increasing $MgCl_2$ concentration, but it is still clearly present at 0.8M $MgCl_2$. An interesting effect in lanes 3–6 occurs where there are protein bands present in the gel; the presence of protein seems to exclude stain altogether, even to the extent of suppressing background staining. This effect is not seen in lanes 1 and 2.

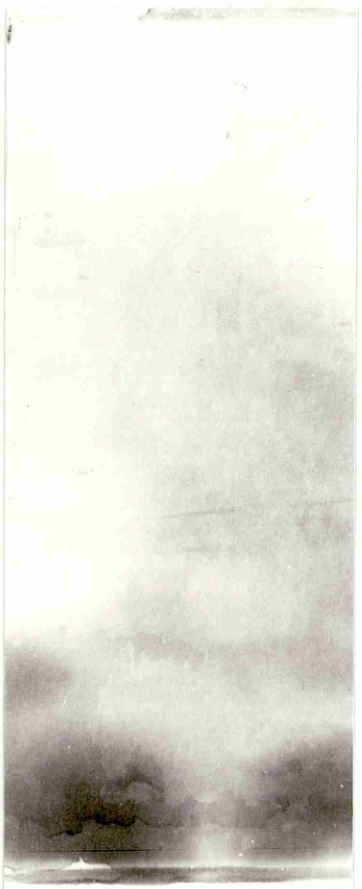


Figure 3.6 Alcian blue staining of bovine corneal extracts using the original staining method. This gel is included for comparison with later gels stained using the CEC technique. There is poor resolution of stained bands due to the very high, patchy background which this method produces.
Lane 1: Molecular weight markers, M_r 205 000, 116 000, 97 400, 66 000, 45 000 and 29 000
Lane 2: 0.15M NaCl extract
Lane 3: 0.15M NaCl plus βmercaptoethanol plus 1%SDS extract

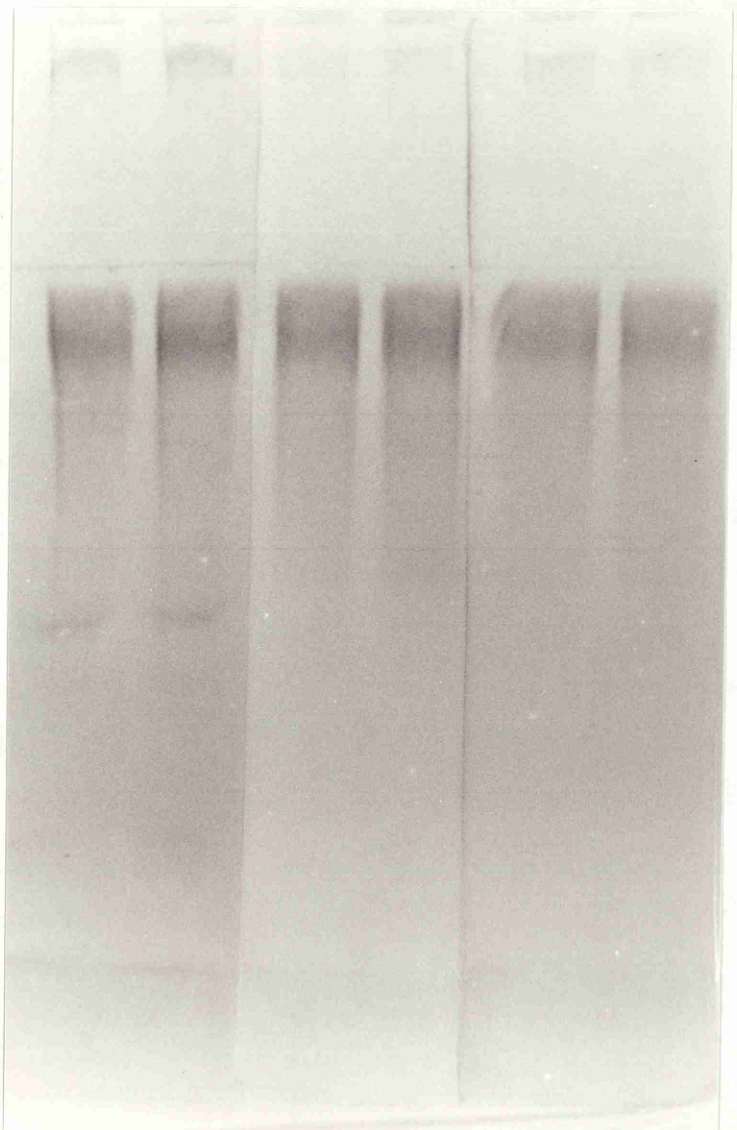


Figure 3.7 Alcian blue staining of bovine corneal extracts under 'critical electrolyte concentration' conditions following SDS-PAGE on 7.5% gel. All lanes contain a NaCl only extract, varying amounts of $MgCl_2$ were added to the stain and destain solutions as shown:
Lanes 1 and 2: No $MgCl_2$ added to stain and destain solutions.
Lanes 3 and 4: 0.2M $MgCl_2$ added to stain and destain solutions.
Lanes 5 and 6: 0.8M $MgCl_2$ added to stain and destain solutions.

3.1.4 Staining of extracts from normal and keratoconus human corneas

Coomassie blue staining of extracts obtained using NaCl and guanidinium chloride

Figure 3.8 compares the pattern of proteins extractable from normal and keratoconus human corneas using 0.15M NaCl or 4M guanidinium chloride. Lanes 1 and 2 compare proteins extractable from normal and keratoconus corneas respectively, in 0.15M NaCl; lanes 3 and 4 show further proteins extractable using 4M guanidinium chloride. Lane 5 contains molecular weight markers. The pattern of proteins extractable from the normal and diseased corneas is very similar, either using 0.15M NaCl or, subsequently, 4M guanidinium chloride. However, there are two proteins of possible interest which differ in the extracts, one is a low molecular weight protein, found only in the normal corneal extracts, seen here running just behind the dye front. The other is a distinctive sharp protein band, of M_r 43 000, seen only in the keratoconus extracts. Less of the proteins of M_r 66 000 and under are apparent in the guanidinium chloride extracts, as these are sequential to extraction in NaCl, however, the harsher extraction treatment does appear to remove more of the higher molecular weight proteins, especially the broad band of staining at M_r 180 000 which corresponds to proteoglycan (see figure 3.9).

Alcian blue staining of extracts obtained using NaCl and guanidinium chloride

Figure 3.9 is the corresponding gel to that shown in figure 3.8, but stained for glycosaminoglycan using Alcian blue. Lanes 1 and 2 compare NaCl extractable proteoglycans from normal and keratoconus corneas respectively. Lanes 3 and 4 contain guanidinium chloride extractable proteoglycans and lane 5 contains molecular weight markers. These markers are visible as they are prestained by the addition of dye conjugated to the marker proteins, they have not taken up Alcian blue stain.

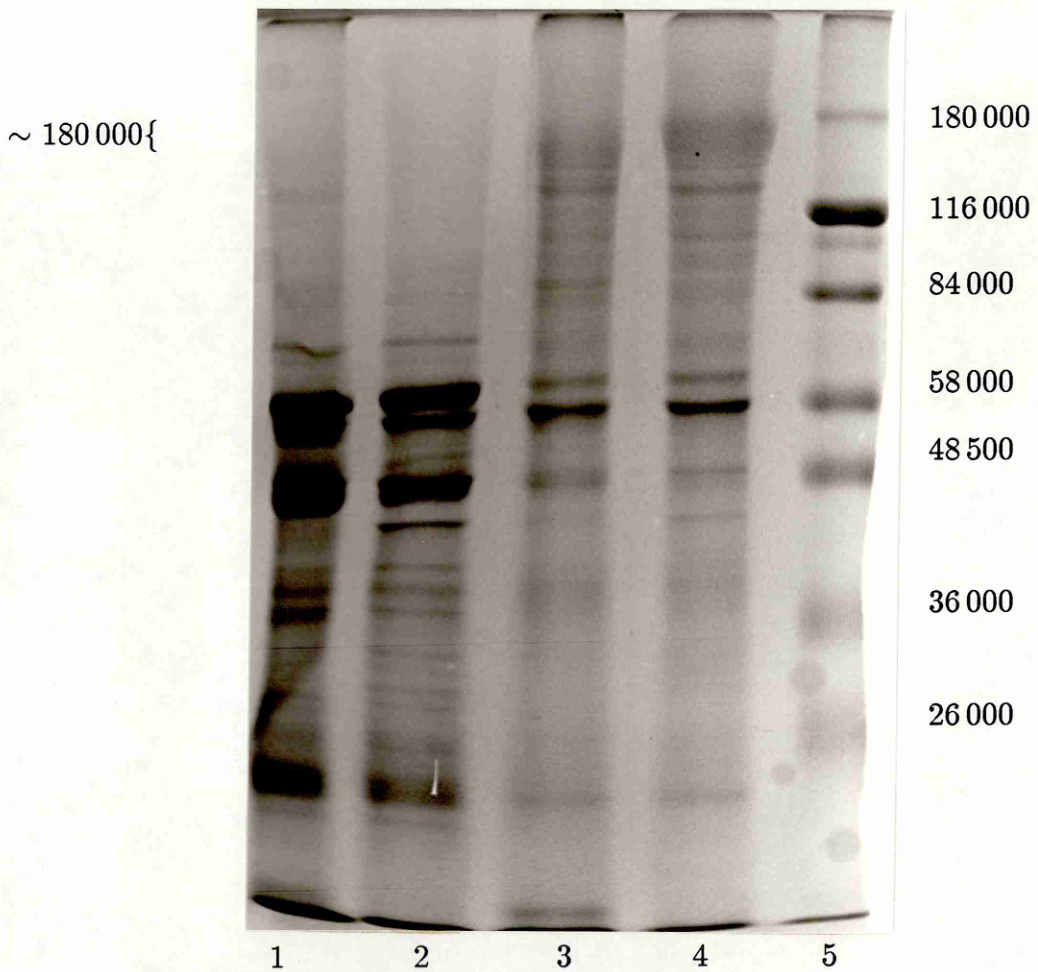


Figure 3.8 Coomassie blue staining of normal and keratoconus human corneas.
Lane 1: Extract from normal human cornea using 0.15M NaCl
Lane 2: Extract from keratoconus human cornea using 0.15M NaCl
Lane 3: Extract from normal human cornea using 4M guanidinium chloride
Lane 4: Extract from keratoconus human cornea using 4M guanidinium chloride
Lane 5: Molecular weight markers, M_r 180 000, 116 000, 84 000, 58 000, 48 500, 36 500 and 26 600

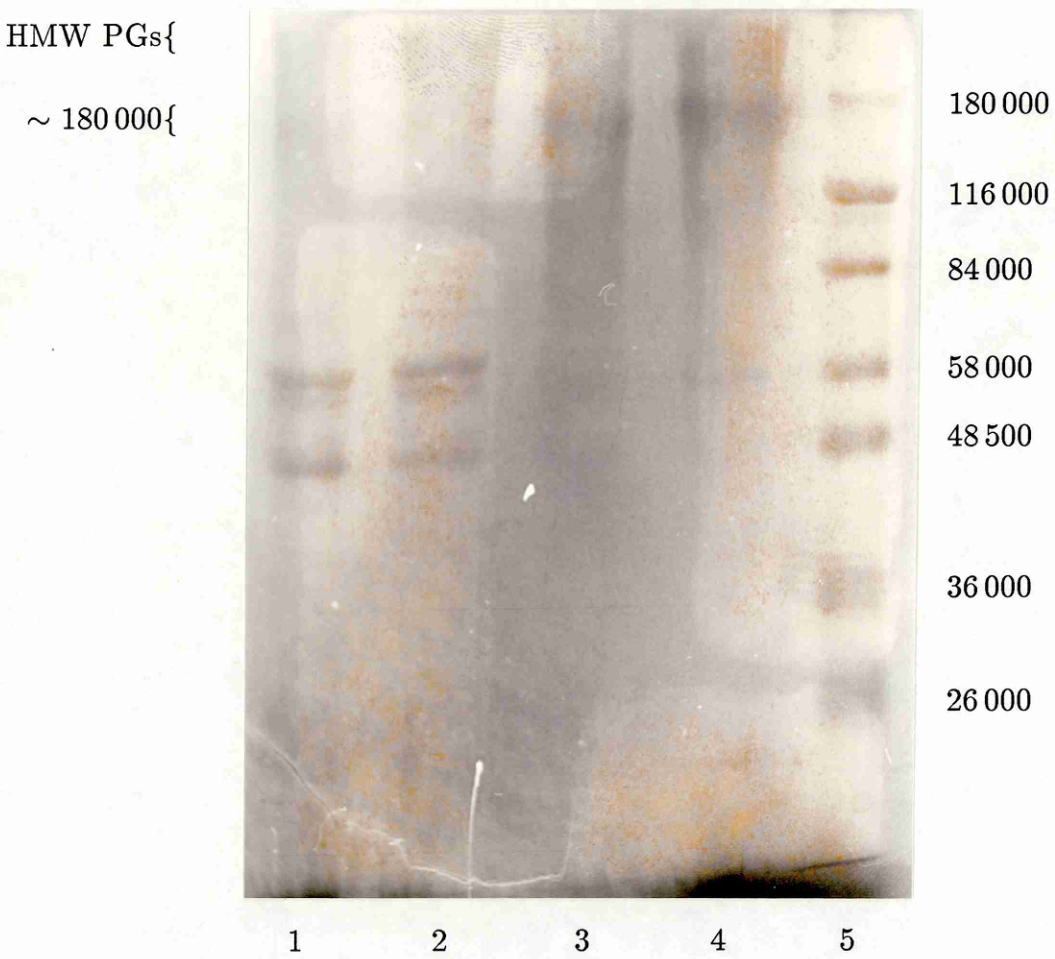


Figure 3.9 Alcian blue staining of normal and keratoconus human corneas.
Lane 1: Extract from normal human cornea using 0.15M NaCl
Lane 2: Extract from keratoconus human cornea using 0.15M NaCl
Lane 3: Extract from normal human cornea using 4M guanidinium chloride
Lane 4: Extract from keratoconus human cornea using 4M guanidinium chloride
Lane 5: Molecular weight markers, M_r 180 000, 116 000, 84 000, 58 000, 48 500, 36 500 and 26 600

There is very little difference between extracts from normal and keratoconus corneas, either using 0.15M NaCl or 4M guanidinium chloride. In the NaCl extracts, staining of components in the 40 000–70 000 range is apparent, with some staining of the high molecular weight polydisperse proteoglycans. In the guanidinium chloride extracts more high molecular weight proteoglycan appears to be present.

The staining characteristics of the polydisperse proteoglycans in these extracts bear similarities to those seen in the extracts from bovine cornea. Protein staining shows one broad band of staining, which in human extracts appears to have a slightly lower molecular weight range than that of bovine (150 000–180 000 as opposed to 180 000–200 000). There is a corresponding area of glycosaminoglycan staining using Alcian blue, plus an additional broad band running slightly behind, but distinct from, the first. This higher molecular weight area of glycosaminoglycan staining is not seen on the gels stained for protein.

3.1.5 Summary of section 3.1

The results obtained from gel electrophoresis of corneal extracts show typical patterns of proteins extractable under different conditions, and indicate the presence of several interesting components, notably BCP54 which was described in the introduction; GP135, a glycoprotein which is extractable from cornea under harsh extraction conditions; and three types of proteoglycan distinguishable on polyacrylamide gels. Two of these proteoglycans are visible in the resolving gel, with molecular weight ranges above 180 000, the third is present in the stacking gel, this proteoglycan having a very high molecular weight which is difficult to estimate in this system. This latter proteoglycan is a very exciting discovery as it suggests the presence of large possibly cartilage-like proteoglycans in normal bovine cornea; their presence has not previously been suggested by other workers.

The development of an efficient staining method for the detection of glycosaminoglycans on polyacrylamide gels has been described, where the addition of $MgCl_2$ to the stain and destain solutions increases the specificity

of staining.

The patterns obtained from normal and keratoconus human corneas are shown, stained for protein and glycosaminoglycan. These show only minor differences in the components extracted, but these may be significant.

3.2 Other biochemical studies

In this section, further work is presented on two of the proteins of interest seen on polyacrylamide gels, BCP54 and GP135. Preparative gel electrophoresis was the method used to prepare pure samples of the two proteins for further work. Amino acid analysis was performed on GP135, in order to compare this with analyses of glycoproteins found in the cornea by other workers. Also, pepsin digestions of corneal extracts were performed, again in order to compare with results obtained by other workers where characteristic fragments of an important corneal glycoprotein, namely type VI collagen, can be seen on polyacrylamide gels following pepsin digestion. Results are also presented on work done to raise antibodies to both BCP54 and GP135, with a view to using these in immunoelectron microscopical studies.

3.2.1 Purification of GP135 and BCP54 by preparative gel electrophoresis

Figure 3.10a shows the stained and destained strip used to locate BCP54 and GP135 after electrophoresis. There is a large amount of undissolved material present at the origin of the gel which has produced streaking of the gel. The streaks occur when the undissolved material present at the origin of the gel slowly goes into solution during running. Gels which ran in this way were regarded as unsuitable for use and discarded.

Figure 3.10b shows an example of the type of gels used to obtain pure samples of BCP54 and GP135. The strip at the left side was stained and destained in order to locate the relevant part of the gel to cut out of the remainder. After the two bands were removed, the rest of the gel was stained and destained in order to check that the correct area had been excised.

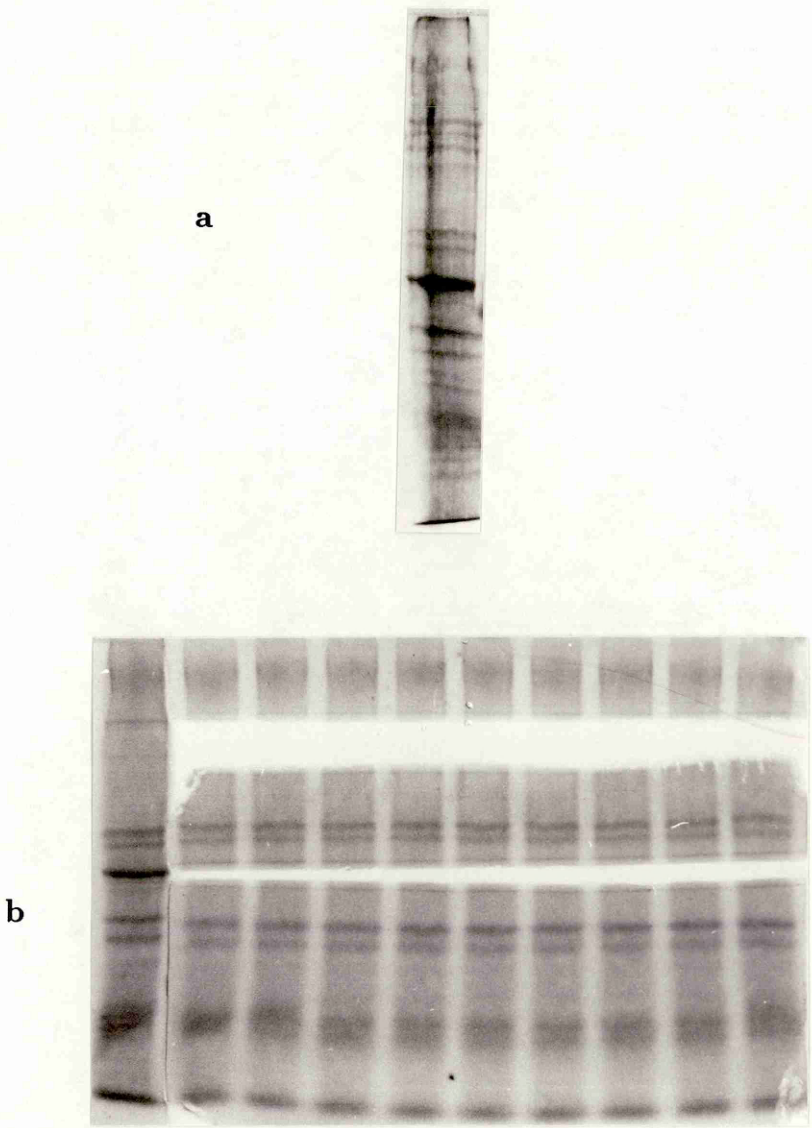


Figure 3.10 Preparative gel electrophoresis of BCP54 and GP135.
a. An unsuitable gel for preparative purposes, due to streaking which has contaminated bands of interest. Gels which ran in this way were discarded.
b. This shows the type of gel used for preparative work. The relevant bands have been removed from the main body of the gel, following alignment with the marker strip which is on the left side of the gel.

3.2.2 Amino acid analysis of GP135

Results of the amino acid analysis of GP135 can be seen in table 3.1.

Amino acid	Residues/1000 residues	Amino acid	Residues/1000 residues
Asp	94	Val	44
Glu	116	Met	6
Hypro	7	Ile	22
Ser	80	Leu	65
Thr	44	Phe	25
Gly	202	Hylys	not determined
Ala	109	Lys	36
Arg	65	His	44
Pro	29	Tyr	12

Table 3.1 Amino acid composition of GP135. Residues are given as residues/1000 amino acids.

Results of the amino acid analysis of GP135 indicate that it cannot be a wholly collagenous protein, as the proportion of glycine is too low (202 residues/1000 as opposed to 333 residues/1000 in type I collagen), also the proportion of hydroxyproline is lower than would be expected. In a later section in the discussion the analysis will be compared with those from other glycoproteins found in the cornea, to see if there are any similarities.

3.2.3 Pepsin digestion of corneal extracts

Figure 3.11 shows the results obtained on polyacrylamide gel electrophoresis following pepsin digestion of a guanidinium chloride extract from bovine cornea. Lanes 1 and 5 contain molecular weight markers. Lane 2 contains pepsin-resistant fragments obtained following digestion of type VI collagen (sample courtesy of Dr. Shirley Ayad). Lane 3 contains the pepsin-resistant fragments from the corneal extract, and lane 4 contains pepsinised type I collagen from calf skin for comparison (Sigma Chemical Co. Ltd., Poole).

There is obviously a large amount of type I collagen in the corneal

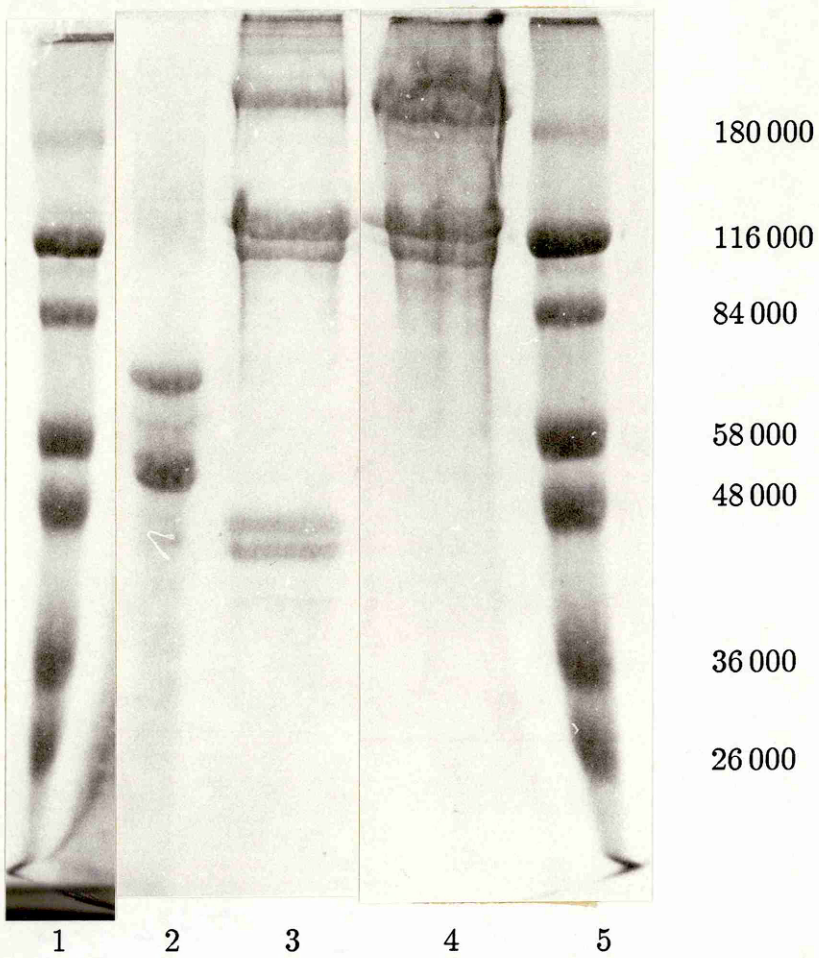


Figure 3.11 Gel electrophoresis following pepsin digestion of bovine corneal extract
Lane 1: Molecular weight markers, M_r 180 000, 116 000, 84 000, 58 000, 48 500, 36 500 and 26 600
Lane 2: Pepsin-resistant fragments from type VI collagen
Lane 3: Pepsin-resistant fragments from bovine corneal extract
Lane 4: Type I collagen from calf skin
Lane 5: Molecular weight markers, M_r 180 000, 116 000, 84 000, 58 000, 48 500, 36 500 and 26 600

extract released by the pepsin treatment, the α1(I), α2(I) and β chains are all clearly present. Further down the gel, there are no bands present which correspond to the pepsin-resistant fragments from type VI collagen; the doublet seen in lane 3 running ahead of the position of the type VI fragments is pepsin.

3.2.4 Western blotting of corneal extracts and testing of antisera

For western blotting and testing the specificity of raised antisera, samples of NaCl, βmercaptoethanol and SDS bovine corneal extracts were separated on polyacrylamide gels and transferred to nitrocellulose paper. Figure 3.12 shows the results obtained following transfer of 4 sample lanes to nitrocellulose. Lane 1 shows the proteins present in a similar extract following gel electrophoresis, for comparison with the proteins obtained following blotting. This is a gel from a different run, therefore the protein bands would not be expected to line up exactly, but do illustrate the extent to which the blotting procedure has worked. Lanes 2 and 3 have been stained for protein, to check blotting efficiency and to locate the positions of the various bands in the extract. Bands of M_r 54 000 and upwards have transferred well onto the nitrocellulose, however bands of lower M_r have not transferred. Lanes 4 and 5 contain the same sample as lanes 2 and 3, and have been used to test the specificity of antiserum raised to BCP54. The two spots at the bottom of lanes 4 and 5 are serum applied directly to the nitrocellulose before the blocking step, in order to check that the visualisation system is working. No staining is present at the position of the M_r 54 000 band.

Figure 3.13 shows the results obtained from two further batches of serum, one raised to BCP54, the other to GP135. Six lanes have been run with corneal extract as before, 2 stained for protein (lanes 3 and 4), 2 used for immunoblotting with BCP54 antiserum (lanes 1 and 2) and 2 used for immunoblotting with GP135 antiserum (lanes 5 and 6). 1 in 2 dilutions of the antisera were used. There is some staining present using

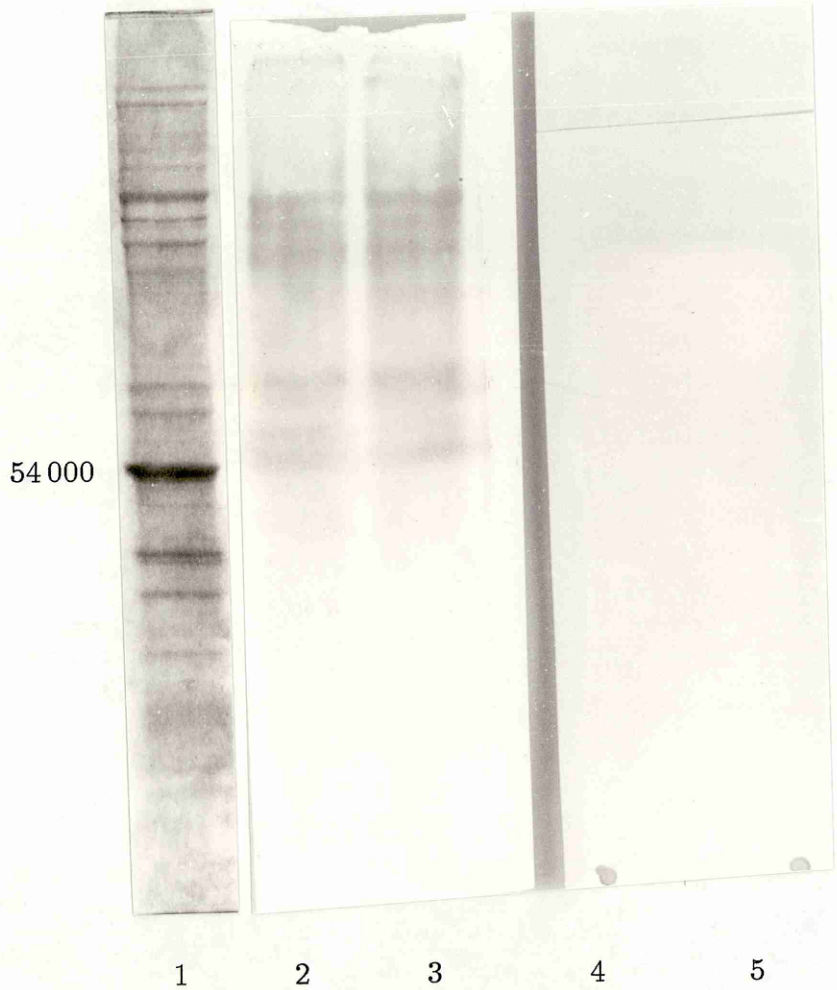


Figure 3.12 Western blotting of corneal extracts and specificity testing of antiserum to BCP54.
Lane 1: NaCl, βmercaptoethanol and SDS corneal extract run on polyacrylamide gel and stained for protein with coomassie blue. This is from a gel in a different run, therefore the bands do not line up exactly with those on the blot. It is included for comparison and to see how well the blotting procedure has worked.
Lanes 2 and 3: NaCl, βmercaptoethanol and SDS corneal extract run on polyacrylamide gel and transferred to nitrocellulose, stained for protein. Bands of M_r 54 000 and upwards have transferred well, lower M_r bands have not transferred.
Lanes 4 and 5: NaCl, βmercaptoethanol and SDS corneal extract run on polyacrylamide gel and transferred to nitrocellulose, used for immunoblotting procedure to check the specificity of antiserum to BCP54. No staining is present at the level of the M_r 54 000 band. The two spots at the bottom of the lanes are present to ensure that the visualisation system is working.

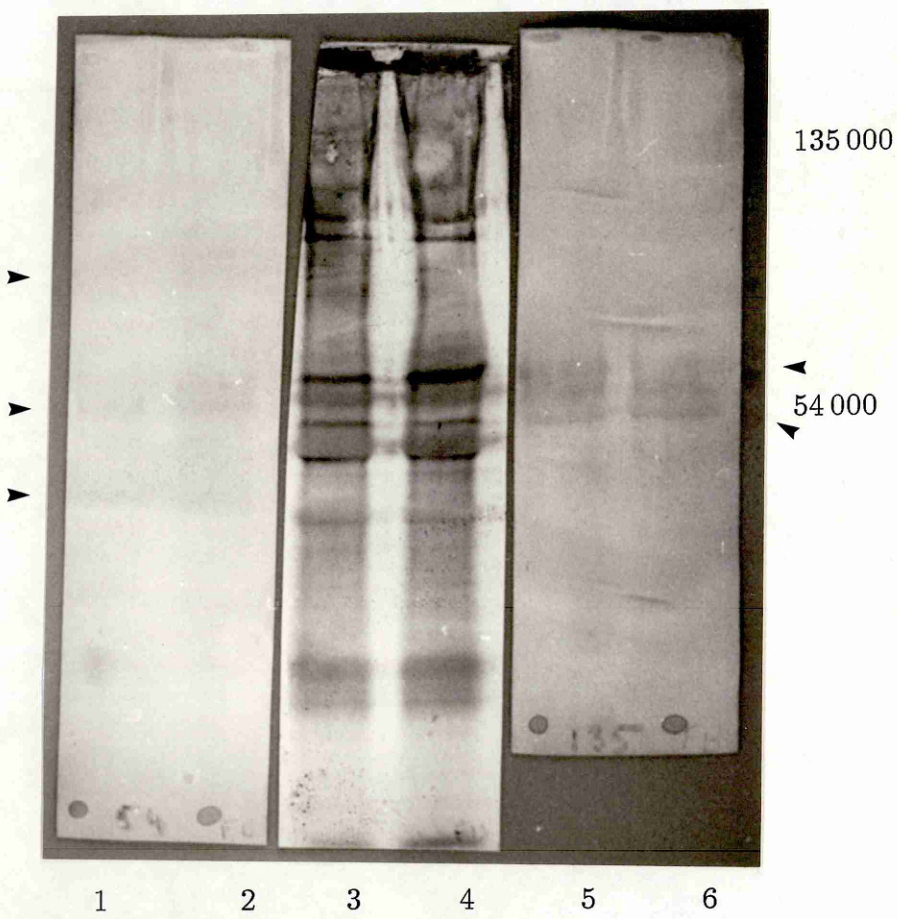


Figure 3.13 Western blotting of corneal extracts and specificity testing of antiserum to BCP54 and GP135.
Lanes 1 and 2: NaCl, βmercaptoethanol and SDS corneal extract run on polyacrylamide gel and transferred to nitrocellulose, used for immunoblotting procedure to check specificity of antiserum to BCP54. Staining is present at the level of the M_r 54 000 band, but additional bands have also stained, indicating that the antiserum is not specific for BCP54.
Lanes 3 and 4: NaCl, βmercaptoethanol and SDS corneal extract run on polyacrylamide gel and transferred to nitrocellulose, stained for protein. Bands of all M_rs have transferred well.
Lanes 5 and 6: NaCl, βmercaptoethanol and SDS corneal extract run on polyacrylamide gel and transferred to nitrocellulose, used for immunoblotting procedure to check specificity of antiserum to GP135. Staining is present at the level of the 54 000, 45 000 and 66 000 M_r bands, but there is no staining at the level of the M_r 135 000 band.

the BCP54 antiserum, as indicated by arrows. There is also staining using the GP135 antiserum, again indicated by arrows, the strongest staining in this case is at the level of the 54 000, 45 000 and 66 000 M_r bands. There is no apparent staining at the level of the 135 000 band. Spots at the bottom of lanes 1,2,5 and 6 again indicate that the visualisation system is working.

3.2.5 Summary of section 3.2

The amino acid analysis of GP135 indicated that it cannot be a wholly collagenous protein as the proportion of glycine is too low, but it remains a possibility that it could contain collagenous areas. In a later section in the discussion the analysis will be compared with those from other glycoproteins found in the cornea, to see if there are any similarities. Pepsin digestion of corneal extracts did not show the characteristic peptic fragments obtainable from type VI collagen, but those of type I collagen were clearly evident. Attempts to raise antibodies to BCP54 and GP135 produced disappointing results, in that the antibodies raised were not specific enough for use in further experiments. The western blotting system worked well to test the specificity of raised antibodies using corneal proteins transferred onto nitrocellulose.

3.3 Electron microscopy of extracted corneas

In this section, electron micrographs will be presented to show the change in the ultrastructure of cornea, following mild and harsh extraction procedures. Micrographs are shown from normal bovine cornea, and those extracted using NaCl or NaCl, βmercaptoethanol and SDS. Also, the negative staining pattern from isolated fibrils following harsh extraction is presented, together with profiles showing the change in stain penetration axially along the collagen fibril following such extraction. Preliminary results following immunogold staining of corneal sections are also shown.

3.3.1 Corneas extracted using NaCl

Following extraction with NaCl, corneas were dried to approximately physiological hydration, using the method described in section 2.2.4, and processed for electron microscopy. Figure 3.15 shows the appearance of the cornea in the electron microscope following this treatment, with figure 3.14 (untreated cornea) also presented for comparison. Sections were positively stained using uranyl acetate. In figure 3.15 the lamellar structure of the cornea can still be seen, although it is not as well defined as in the untreated corneas, and fibrils cut in transverse section show a degree of disorder and occasional aggregation. Additionally, the centres of a large number of the fibrils cut in transverse section have not taken up stain. Fibrils seen in longitudinal section have taken on a more 'wavy' appearance than those of untreated cornea, however, their axial banding pattern appears similar to untreated cornea. The disordering of the structure has led to the appearance of spaces between the fibrils which can be seen in both the transverse and longitudinal areas. In figure 3.14 close examination shows the presence of a fine network of material which has taken up stain in the interfibrillar spaces, whereas this material does not appear to be present in figure 3.15.

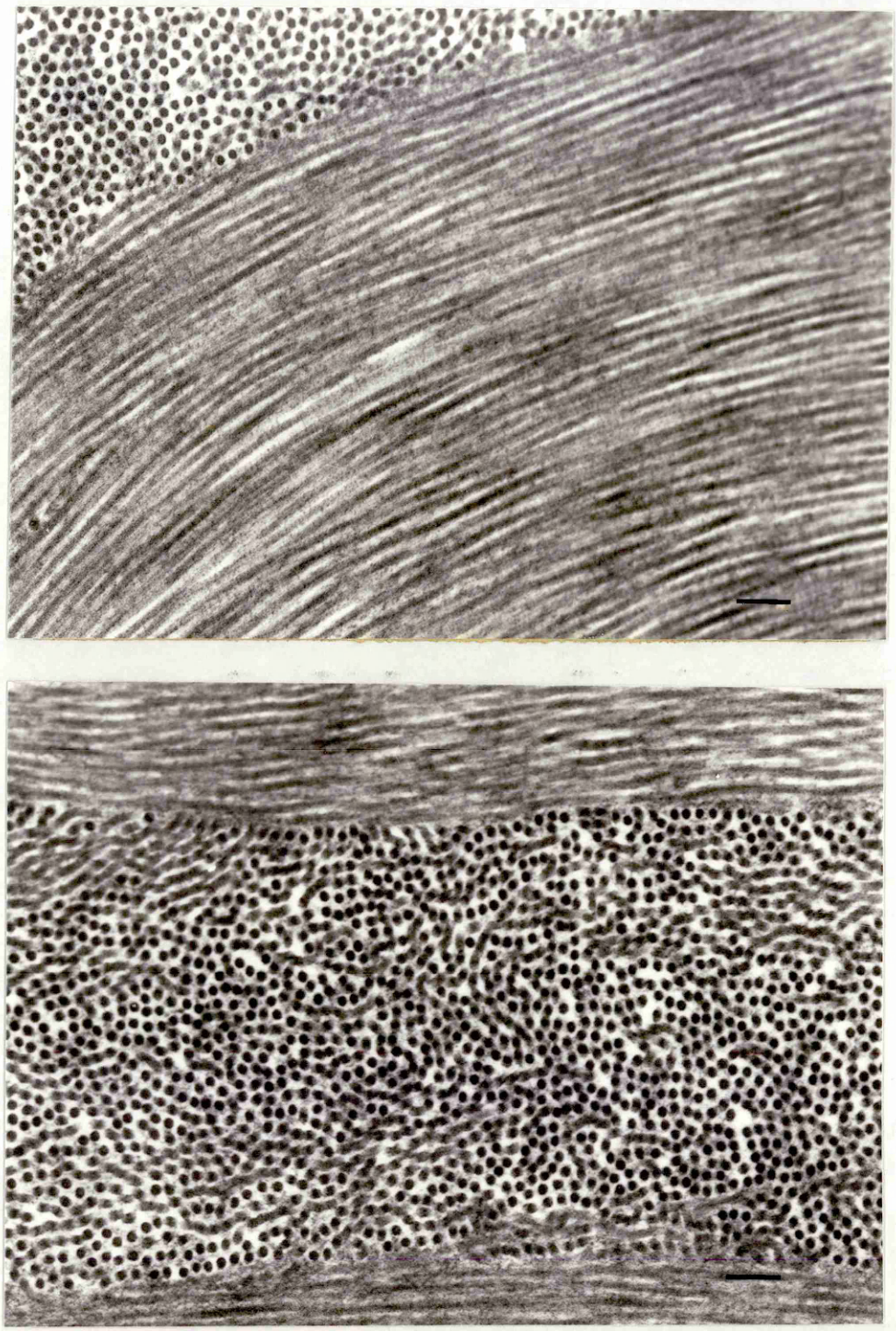


Figure 3.14 Electron micrographs of untreated bovine cornea. Sections were positively stained using uranyl acetate. The distinctive lamellar structure and regular fibril spacing can be seen. Bar=200nm
Micrograph courtesy of Dr. P. Cooke

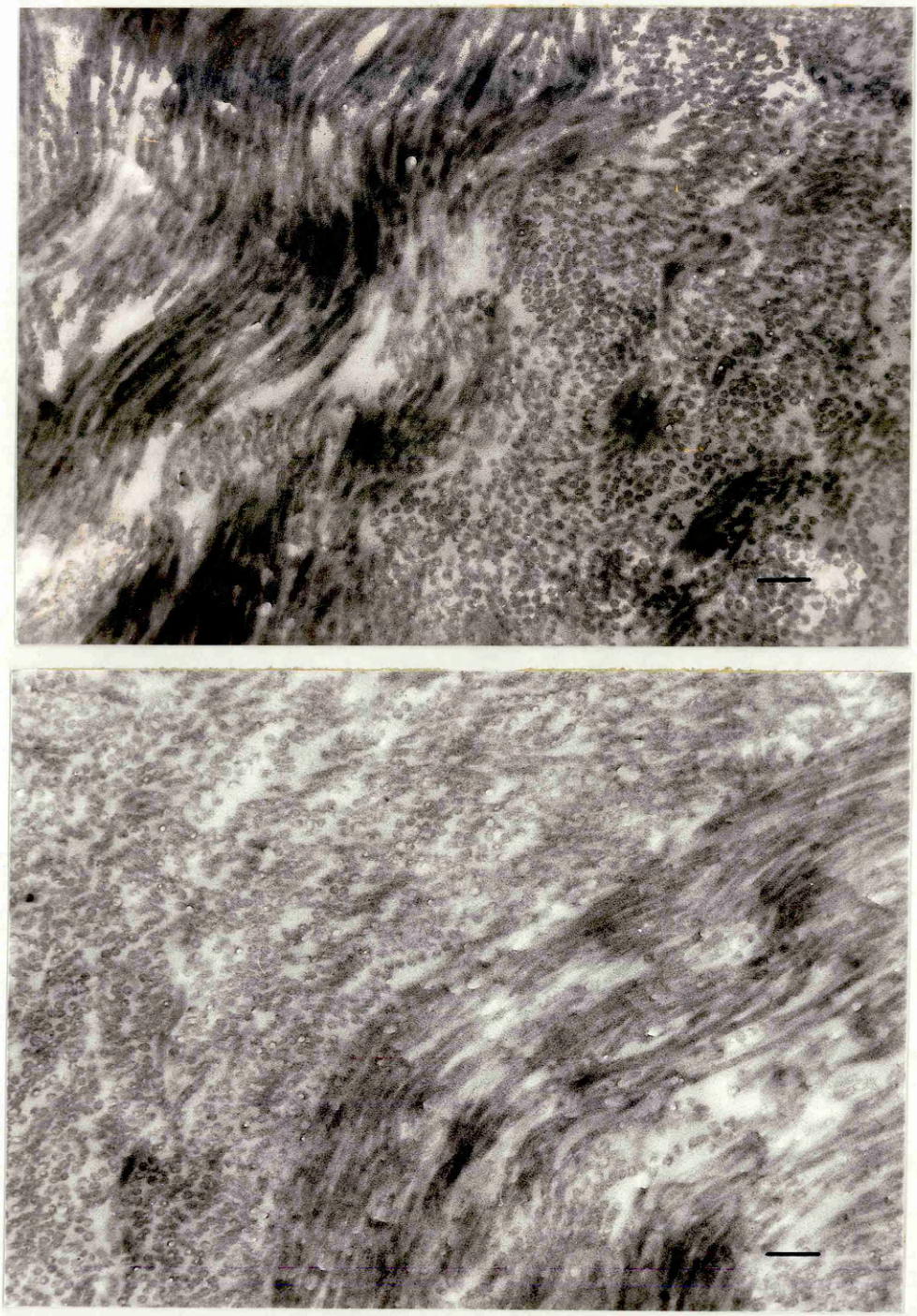


Figure 3.15 Electron micrographs of bovine cornea following extraction with NaCl. Sections were positively stained using uranyl acetate. Mild disruption of the stromal organisation can be seen, also spaces in the structure resulting from this. Bar=200nm

3.3.2 Corneas extracted using NaCl, βmercaptoethanol and SDS

Following extraction with NaCl, βmercaptoethanol and SDS, corneas were processed as described previously. Figure 3.16 shows the appearance of corneas following this treatment. In this case the stromal disruption is so severe that the lamellar structure is not distinguishable. Fibrils cut in transverse section show gross disorder and aggregation, with large spaces appearing in the structure. Fibrils seen in longitudinal section appear to stick together in broad sheets over large areas, and the axial banding pattern is much more distinctive than in those extracted with salt alone. Where the fibrils have aggregated the bands are in register with each other, and again, large spaces can be seen between the groups of aggregated fibrils.

The disruption and other effects seen here are typical when chaotropic agents are used to extract corneal tissues. Monkey cornea extracted using 4M guanidinium chloride shows a very similar alteration in ultrastructure [Gyi, 1988]. This is an important point, and indicates that the effects of SDS or guanidinium chloride extraction on the tissue are similar, at least with respect to their effect on ultrastructure.

3.3.3 Negative staining of corneas extracted using NaCl, βmercaptoethanol and SDS

Figure 3.17 shows the results obtained following negative staining of fibrils isolated from corneas extracted using NaCl, βmercaptoethanol and SDS. Figure 3.17a shows an electron micrograph of the negatively stained fibrils. The banding pattern can be seen along the fibril, although it is not very clearly defined. Figure 3.17b shows the axial pattern of stain exclusion obtained from negatively-stained untreated corneal collagen. Figure 3.17c shows the axial pattern of stain exclusion obtained from negatively-stained corneal collagen following extraction of corneas using NaCl, βmercaptoethanol and SDS. Figures 3.17b and 3.17c were obtained by averaging densitometric scans of several D-periods of corneal collagen from electron micrograph

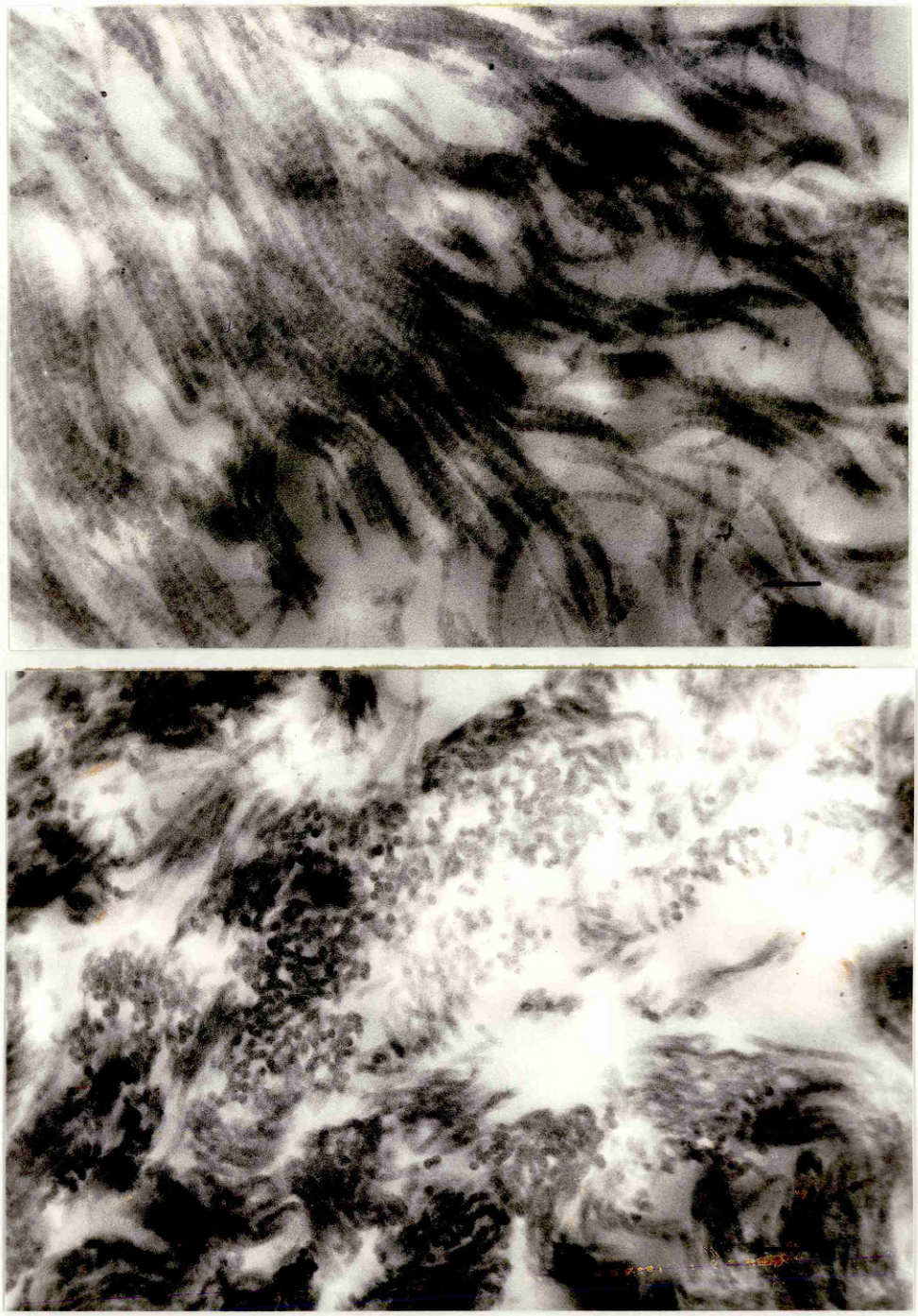


Figure 3.16 Electron micrographs of bovine cornea following extraction with NaCl, βmercaptoethanol and SDS. Sections were positively stained using uranyl acetate. Severe disruption of the stromal organisation can be seen, and large spaces have appeared in the structure. Bar=200nm

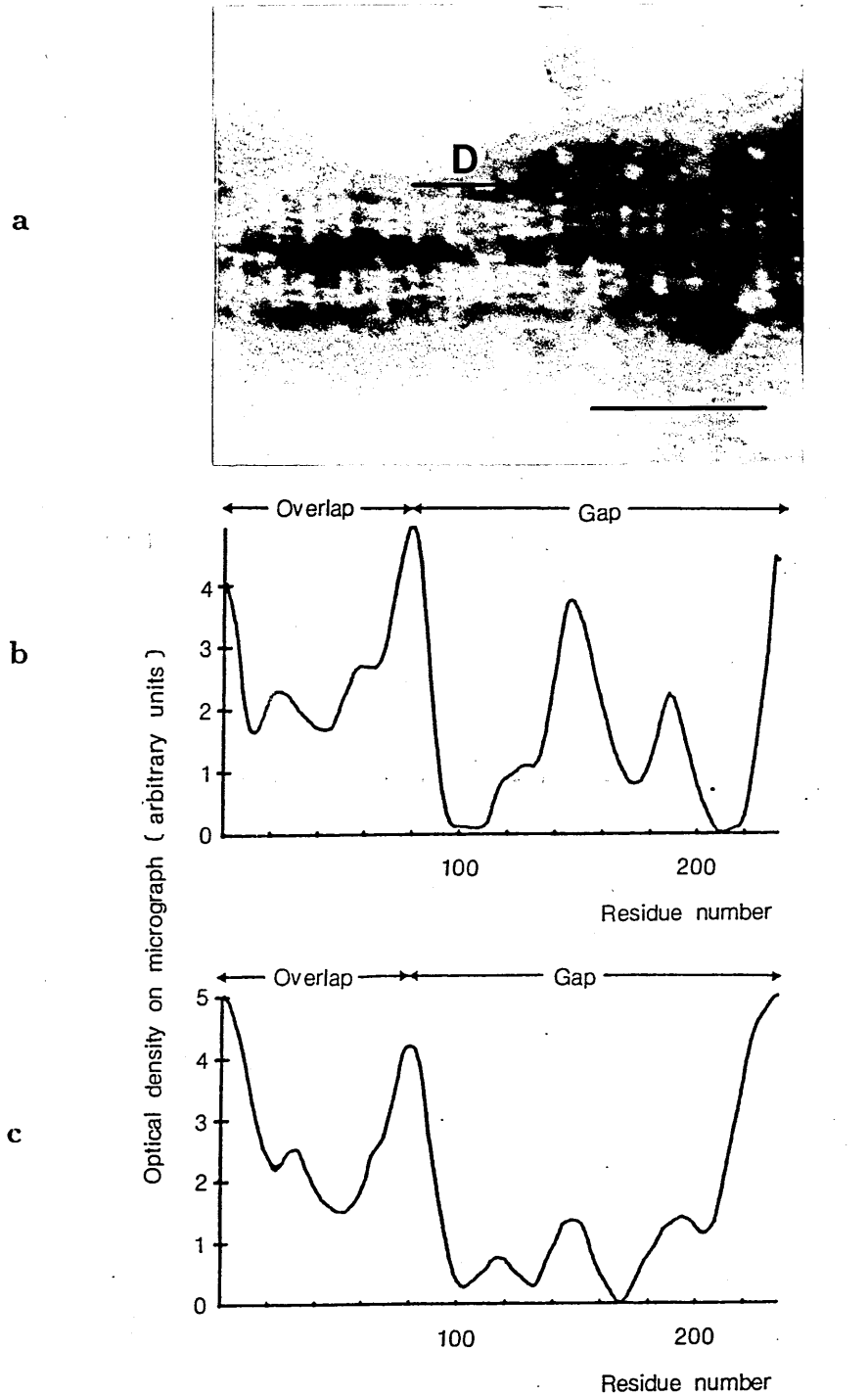


Figure 3.17 Negative staining of collagen following extraction of corneas using NaCl, βmercaptoethanol and SDS.
a. Electron micrograph of negatively-stained corneal collagen fibril following extraction using NaCl, βmercaptoethanol and SDS. Bar=100nm.
b. Stain exclusion by untreated corneal collagen, obtained from electron micrographs of negatively-stained material.
c. Stain exclusion by NaCl, βmercaptoethanol and SDS-extracted corneas, obtained from electron micrographs of negatively-stained material.

negatives as described in section 2.5.3; they are broadly similar in the overlap region, the gap region of the extracted corneas shows a reduction in the amount of material present.

3.3.4 Immunogold staining of normal bovine corneal sections

Preliminary experiments were carried out to see if the antibodies raised to corneal proteins could be used to locate the proteins following immunogold staining of bovine corneal sections embedded in lowicryl K4M. Western blotting techniques showed that the antibodies used were unfortunately not specific enough to label the proteins of interest, however in principle immunogold staining would be a good method to locate proteins within the corneal stromal framework given suitable antibodies.

3.3.5 Summary of section 3.3

The electron micrographs of extracted corneas clearly show increasing disruption to the ultrastructure with more harsh extraction methods. An important effect seen in those corneas extracted using SDS is the aggregation of collagen fibrils, suggesting the removal of a component or components which would normally space the fibrils from one another. The axial banding pattern is much more distinctive, suggesting removal of collagen-associated material by this method. Also, the optical density profile from micrographs of negatively-stained extracted collagen shows a dramatic decrease in material present at the gap region of the collagen fibrils.

3.4 X-ray diffraction results

As stated in the introduction, X-ray diffraction can be used to study collagenous tissues, as these exhibit regularity of structure over a limited size range. The low-angle meridional X-ray diffraction pattern from bovine corneal stroma is unusual when compared with the pattern from other collagen-containing tissues such as tendon, in that the first order reflection is missing. In tendon, the first order is by far the most intense reflection, and arises principally from the step in electron density [Tomlin and Worthington, 1956] which occurs at each gap/overlap junction along the collagen fibrils [Hodge and Petruska, 1963]. The absence of the first order reflection in the pattern from bovine cornea is thought to be due to the presence of additional material occupying a large part of the gap region [Meek *et al.*, 1981a,Meek *et al.*, 1981b]. The nature of this material will be discussed more fully in chapter 4. The results presented here compare the diffraction pattern obtained from bovine corneal stroma following harsh extraction procedures with that of normal cornea and tendon.

3.4.1 Corneas extracted using NaCl, βmercaptoethanol and SDS

Figure 3.18a shows the low-angle X-ray diffraction pattern obtained from corneas following their extraction in NaCl plus βmercaptoethanol. This is a short exposure picture, taken to record the lower orders of the diffraction pattern. No first order reflection is present in this pattern. Figure 3.18b shows the pattern from corneas following their extraction in NaCl plus βmercaptoethanol plus SDS. In this pattern the first order reflection is clearly evident. Longer exposures of these samples show the higher orders of the meridional pattern (results not shown).

Neither of these patterns show the presence of an equatorial ring, which in untreated cornea arises from the regular arrangement of the collagen fibrils. The absence of this pattern indicates that some degree of order has been lost.

a

b

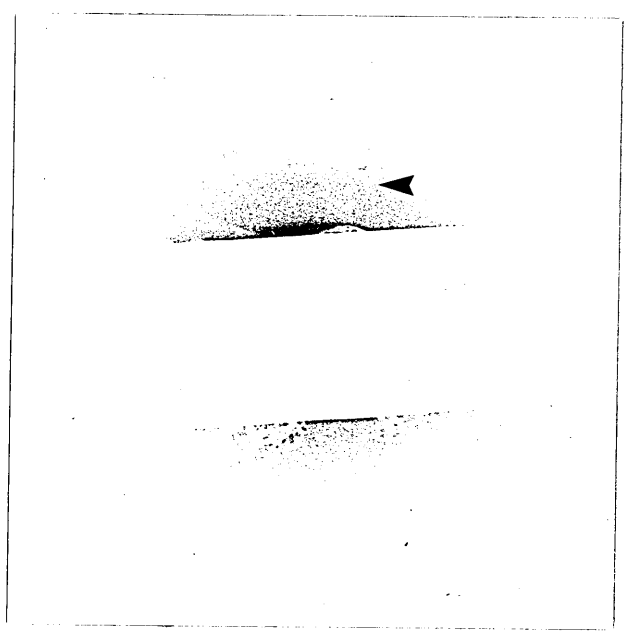


Figure 3.18 X-ray diffraction patterns from extracted bovine corneas.
a. Corneas extracted using NaCl plus βmercaptoethanol
b. Corneas extracted using NaCl plus βmercaptoethanol plus SDS. The first order meridional reflection is indicated by an arrow.

3.4.2 Corneas extracted using NaCl and guanidinium chloride

Figure 3.19 shows the X-ray plates obtained from corneas following extraction in 4M guanidinium chloride. Figure 3.19a is a short exposure picture which records the lower orders of the meridional diffraction pattern, the first and third orders are most clearly seen here; figure 3.19b is a longer exposure picture taken to record the higher orders of the pattern. From these two patterns, densitometer scans were taken to obtain the relative intensities of the different orders of the patterns.

Order	GuCl extracted bovine cornea	Tendon(†)	Normal bovine cornea(‡)	Phases(§)
1	770	6357	0	66.2
2	187	172	0	90.6
3	693	693	693	-6.5
4	0	70	38	56.0
5	385	343	312	-69.9
6	54	108	51	-123.2
7	156	134	25	-166.0
8	84	38	57	-18.6
9	230	451	57	39.5
10	81	121	19	-165.0

Table 3.2 Relative intensities of the first ten orders of the meridional diffraction pattern for normal and extracted corneas and tendon
†Taken from Hulmes *et al.*, (1977)
‡Taken from Meek *et al.*, (1981a)
§Taken from Hulmes *et al.*, (1980). The phases given here refer to a phase origin of 0.

Table 3.2 shows the integrated intensities of the meridional pattern from corneas extracted using 4M guanidinium chloride (1 day extracts), compared with data from tendon [Hulmes *et al.*, 1977] and normal bovine cornea [Meek *et al.*, 1981a]. These data have been arbitrarily scaled together by equating the third order intensities. This procedure minimises the differences for most of the diffraction orders. With this scaling, the most

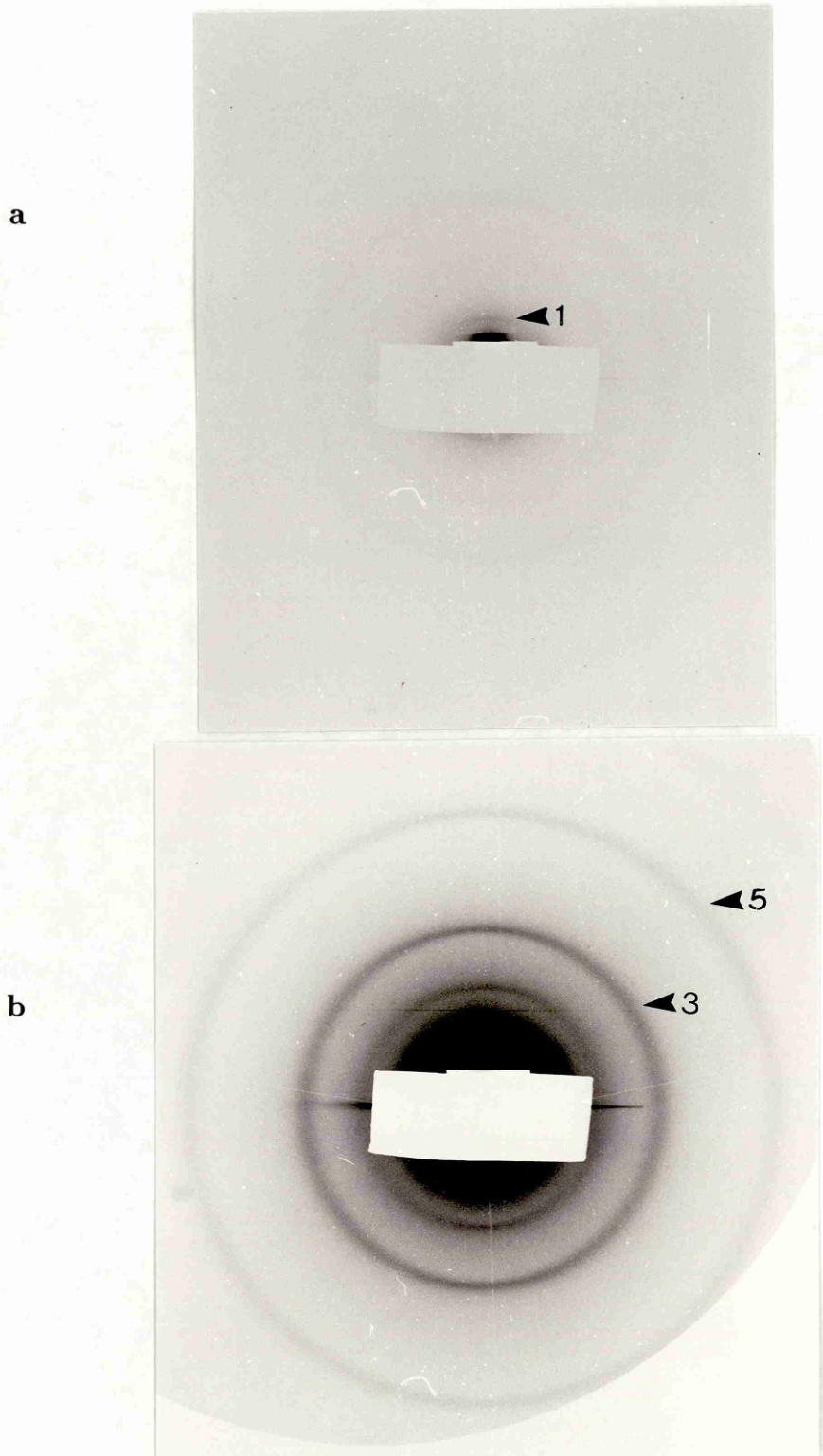


Figure 3.19 X-ray plates of meridional diffraction pattern from guanidinium chloride extracted cornea
a: Short exposure to obtain the lower orders of the diffraction pattern.
b: Longer exposure to obtain the higher orders of the diffraction pattern.
The first, third and fifth orders of the meridional pattern are indicated on the photographs.

striking differences in intensity can be seen in the first and ninth orders of the pattern, where intensity values for extracted cornea are between those of normal cornea and tendon. This table also shows the phases used in the calculation of axial electron density, taken from Hulmes *et al.*, (1980).

3.4.3 Electron density profiles from meridional X-ray diffraction patterns

Figure 3.20 shows the computed electron densities for normal bovine cornea (3.20a), guanidinium chloride extracted cornea (3.20b) and tendon (3.20c). The distribution of electron density in the extracted cornea appears to be intermediate between that of untreated cornea and tendon, suggesting the removal of components from the corneal collagen such that it becomes more like that of tendon. In the overlap region, the electron density distribution of extracted cornea looks very similar to tendon, and in the gap region the relative amount of material present has been reduced in the extracted cornea, and more clearly defined peaks in electron density have become apparent. Comparison of the electron density distributions in this way does not involve scaling used to compare sets of intensity data, and thus provides a good method to assess the changes in structure to the hydrated corneal collagen caused by the extraction procedure.

3.4.4 Summary of section 3.4

The results obtained from X-ray diffraction experiments are in agreement with those from electron microscopy, in that they suggest similar structural alterations in extracted corneas. The absence of the interfibrillar diffraction pattern indicates loss of fibril packing order in extracted corneas, this can also be seen very clearly in electron micrographs. The appearance of the first order of the meridional pattern in extracted corneas suggests removal of material at the gap regions of the collagen fibrils. The electron density distribution computed from the relative intensities of the different orders of the meridional pattern is also consistent with removal of material from the

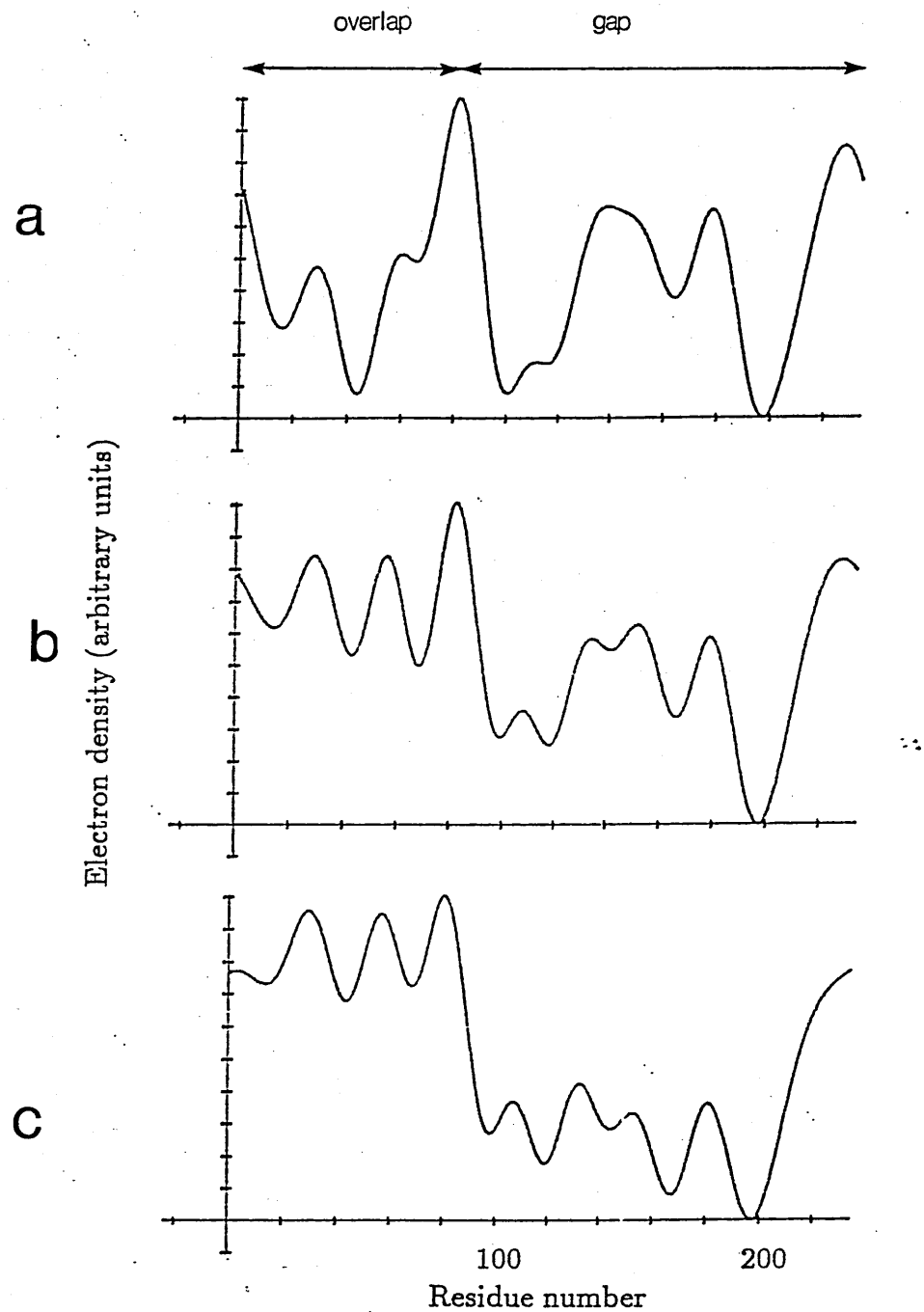


Figure 3.20 Axial electron density distribution of collagen fibrils in normal and extracted bovine cornea and tendon
a: Normal bovine cornea
b: Bovine cornea following extraction in 4M guanidinium chloride
c: Tendon

gap region in extracted corneas, and also indicates some structural changes occurring in the overlap region.

3.5 Transparency measurements from extracted corneas

In this section, the effect of guanidinium chloride extraction on the transparency of cornea is examined. Section 2.7.1 in the methods describes the procedure used to extract corneas prior to measurement of their absorbance. Briefly, bovine corneas were extracted for 3 days initially in 0.15M NaCl, and then divided into two groups, one group continued to be extracted in 0.15M NaC for 1,3 and 5 days, the other group was extracted in 0.15M NaCl plus 4M guanidinium chloride, in order to see the effect of guanidinium chloride in the extraction solution. The corneas were scanned using a laser densitometer, such that an increase in absorbance indicated a decrease in transparency.

3.5.1 Measurement of absorbance values from densitometer traces

Figures 3.21 and 3.22 are examples of the densitometer scans used to measure the absorbance values for extracted corneas. The scans have different absorbance scales, in order to measure the absorbance values more accurately. Figure 3.21a is a scan from one of the guanidinium chloride extracted corneas; the background absorbance can be easily assessed, and an absorbance value for the swollen cornea obtained fairly accurately. Figure 3.21b is a corresponding densitometer trace for the guanidinium chloride extracted corneas, but after the extraction solution has been washed out using 0.15M NaCl; it is clear that there is a large increase in absorbance, and hence decrease in transparency, once the guanidinium chloride has been removed from the cornea. Figure 3.21c shows a scan from a cornea extracted in 0.15M NaCl alone, this has a much higher absorbance than corneas where guanidinium chloride is included in the extraction solution. Following an equivalent salt wash, figure 3.21d shows a high absorbance value, similar to that of figure 3.21b.

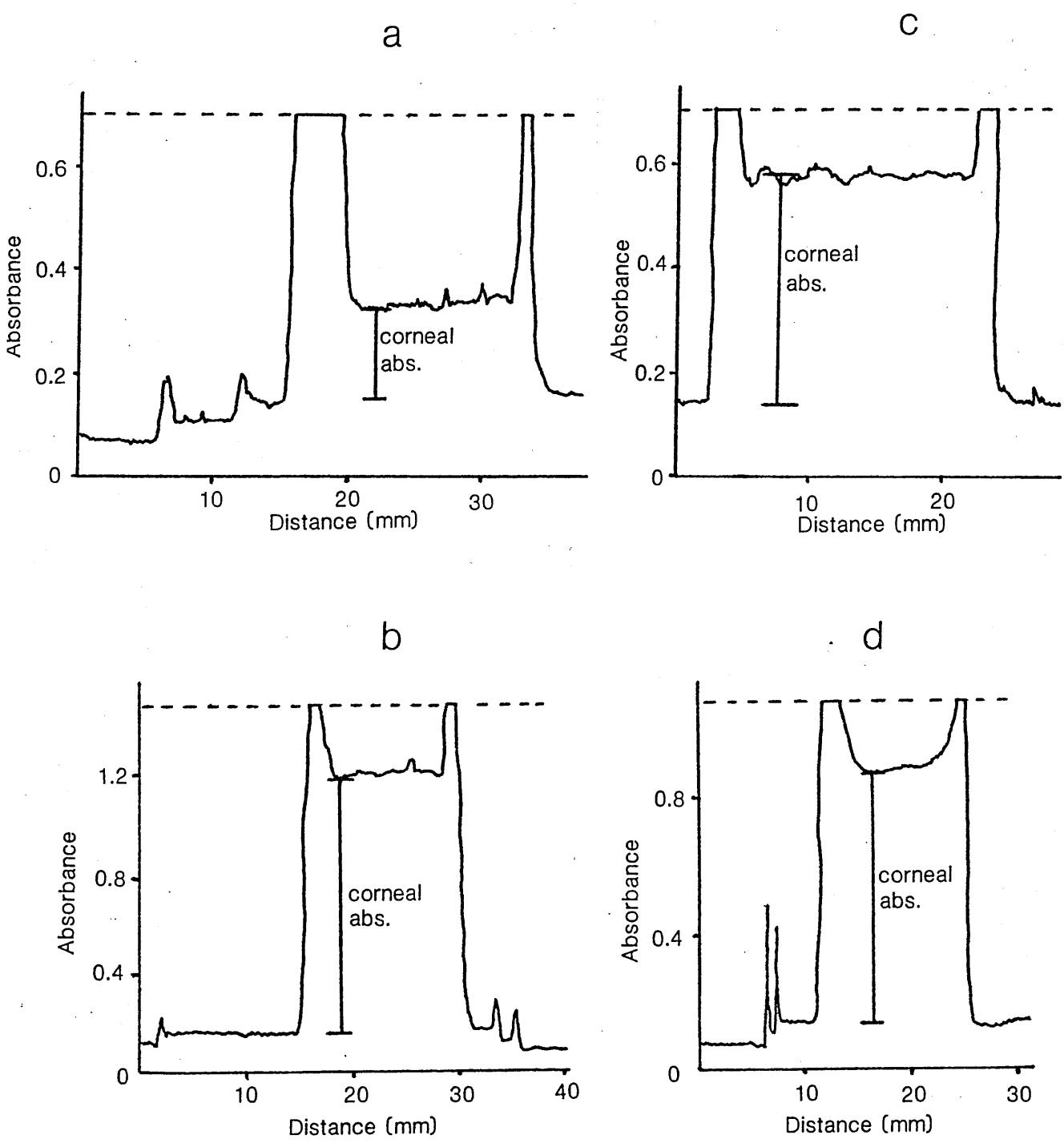


Figure 3.21 Laser densitometer scans of swollen, extracted bovine corneas
a: Corneas extracted for 1 day in 4M guanidinium chloride plus 0.15M NaCl
b: Corneas washed for 4 x 1 day in 0.15M NaCl after extraction as in a.
c: Corneas extracted for 1 day in 0.15M NaCl
d: Corneas washed for 4 x 1 day in 0.15M NaCl after extraction as in c.

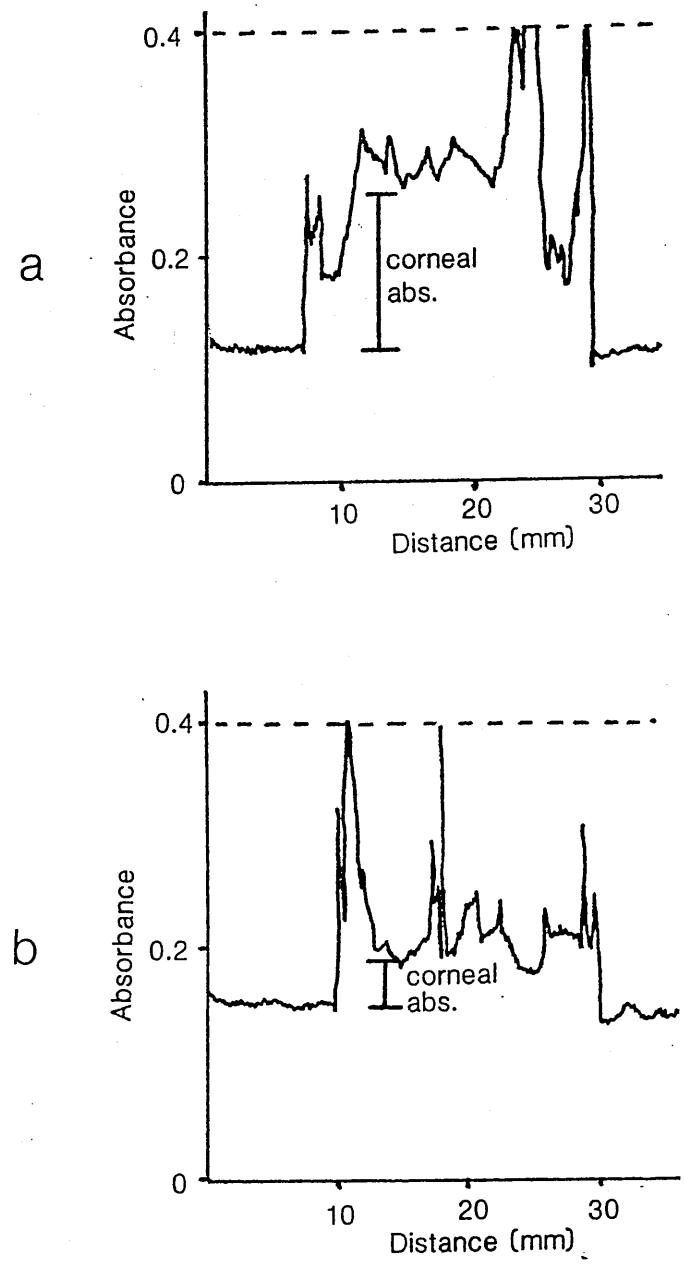


Figure 3.22 Laser densitometer scans of extracted bovine corneas following drying to physiological hydration
a: Corneas extracted for 1 day in 4M guanidinium chloride plus 0.15M NaCl, followed by washing in 0.15M NaCl, then drying.
b: Corneas extracted for 1 day in 0.15M NaCl, followed by washing in 0.15M NaCl, then drying.

Densitometer scans are also shown from extracted corneas after they have been dried down to approximately physiological hydration (figure 3.22). These scans show considerably more noise than those from swollen corneas, due to the difficulty of obtaining a flat, unwrinkled surface on the top and bottom of the corneas, however, they do show that a large proportion of the corneas' original transparency is regained, both by corneas extracted in 0.15M NaCl alone, or by corneas extracted in 4M guanidinium chloride plus 0.15M NaCl.

Table 3.3 (page 97) gives the actual absorbance values obtained from corneas at various stages in the extraction procedure. It is clear that continuing extraction in NaCl leads to higher absorbances. Transferring corneas from 0.15M NaCl solution to extraction solution containing guanidinium chloride produces a substantial drop in absorbance, from 0.35 down to 0.18, thus the transparency of swollen corneas is considerably improved in guanidinium chloride solution. However, this is a reversible situation, as washing out the guanidinium chloride solution from the corneas produces an increase in absorbance, up to 1.01, which is of the same order as that from corneas extracted in NaCl alone. Following drying, corneal transparency is considerably improved, although not to the same level as fresh, unextracted cornea. This indicates that the components which have been removed by the extraction procedure are not essential for transparency *per se*, but they may be involved in the maintenance of those aspects of the structure which are important for transparency (e.g. control of fibril diameter).

3.5.2 Summary of section 3.5

It is well known that swelling of cornea leads to a decrease in transparency. The results presented here indicate that there can be a large variation in the transparency of swollen corneas, depending on the type of solution that the corneas are immersed in. The presence of 4M guanidinium chloride considerably improves the transparency of swollen corneas when compared with corneas extracted in 0.15M NaCl. Subsequent removal of the guanidinium chloride results in substantial loss of transparency. Following drying to ap-

Specimen	Absorbance	n
Fresh cornea	0	1
Corneas extracted for 3 days in NaCl(swollen)	0.35 ± 0.04	21
Corneas extracted for 3 days in NaCl(dry)	0.045 ± 0.007	3

Specimen	Absorbance	n	Specimen	Absorbance	n
Corneas extracted for 1 day in GuCl(swollen)	0.18 ± 0.03	9	Corneas extracted for 1 day in NaCl(swollen)	0.44 ± 0.04	9
As above following 4 days NaCl wash(swollen)	1.01 ± 0.05	3	As above following 4 days NaCl wash(swollen)	0.79 ± 0.02	3
As above after drying	0.13 ± 0.02	3	As above after drying	0.07 ± 0.03	3
Corneas extracted for 3 days in GuCl(swollen)	0.14 ± 0.03	6	Corneas extracted for 3 days in NaCl(swollen)	0.69 ± 0.08	6
As above following washing & drying	0.22	1	As above following washing & drying	0.065 ± 0.008	2
Corneas extracted for 5 days in GuCl(swollen)	0.14 ± 0.01	3	Corneas extracted for 5 days in NaCl(swollen)	0.82 ± 0.08	3
As above following washing & drying	0.32 ± 0.14	3	As above following washing & drying	0.07 ± 0.03	3

Table 3.3 Absorbance values for extracted corneas when swollen, following washing, and following drying to approximately physiological hydration.

proximately physiological hydration, the transparency of both guanidinium chloride- and NaCl-extracted corneas approaches that of fresh cornea.

Chapter 4

Discussion

4.1 The material present in the gap regions of corneal collagen fibrils

Corneal collagen fibrils differ from those found in other connective tissues in several respects, e.g. the uniformity and small size of the fibrils, and differences in the structure of the fibrils at the gap region. This difference in structure can be observed as an alteration in the negative banding pattern of corneal compared with other type I collagen fibrils, and, using X- ray diffraction, by a difference in the axial electron density profile of corneal collagen which indicates increased electron density at the gap region.

There are several structural possibilities which would account for this difference. Figure 4.1a shows the structure of tendon collagen fibrils for comparison, with an obvious space between the tail of one collagen molecule and the head of the next, resulting in the decreased electron density found in the gap region.

Figure 4.1b shows one possible model to account for the structural difference in corneal fibrils; a covalent head or tail extension to each individual collagen molecule which would fill the space normally found in the gap region, and thus even out the axial electron density. If this were the case, then extraction methods designed to remove electrostatically bound proteins would not be expected to have any effect on the gap/overlap elec-

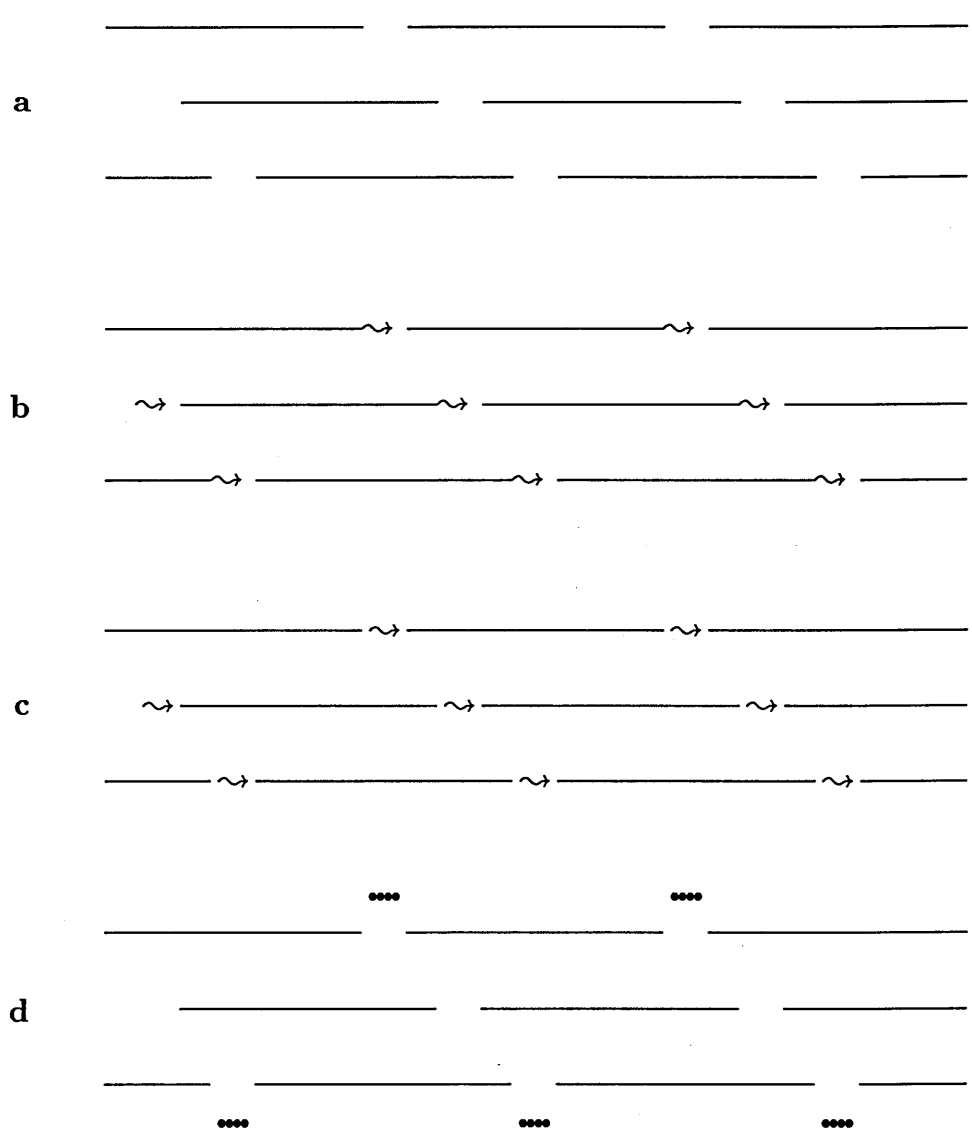


Figure 4.1 Models to account for the difference in electron density in corneal compared to tendon collagen.
a: Diagrammatic model of tendon collagen fibrils, with a gap between the head of one collagen molecule and the tail of the next.
b: Model showing a covalent extension (⇝) on the head of each collagen molecule (a similar model would have the extension attached at the tail end.)
c: Model showing collagenous fill-in molecules (⇝) in each of the gap regions.
d: Model showing non-collagenous proteins associated with the collagen externally at the level of the gap regions.

tron density profile, as the covalent extensions would remain *in situ*. It is possible that this elongated type of collagen could escape detection using standard methods of preparation such as pepsin digestion, if the extension was susceptible to pepsin and was degraded during the process. However, in order for the extensions to fit into the available spaces without disrupting the packing of the collagen molecules, it would be reasonable to assume that they themselves must be triple- helical, and thus collagenous in structure. This would confer pepsin resistance to them, which would result in the preparation of unusually large collagen molecules. As no such collagens have been discovered in cornea this model is unlikely to be correct.

An alternative model shown in figure 4.1c shows protein molecules filling in the individual spaces between the collagen molecules. As discussed above these proteins might be expected to be collagenous in order to fit into the structure without causing disruption. The length of each collagen molecule is approximately 300nm with M_r of 300 000 for the three polypeptide chains. The length of the gap is approximately 40nm, thus the maximum M_r of the fill-in collagen would be 40 000 in total, with individual polypeptide chains of approximately 13 000 M_r. If these were not covalently linked to the rest of the fibril structure then it should be possible to remove them by extraction, and thus alter the electron density profile of the fibrils to become more like that of tendon. While harsh extraction methods do produce such a change in the electron density profile, as can be seen in figures 3.17 and 3.20, no small collagenous molecules have been found in the corneal extracts, so this model is unlikely to account for the observed differences.

A third model is shown in figure 4.1d. An additional protein associates with the collagen fibrils externally at the level of the gap region. In order to alter the electron density profile to the extent necessary, the protein would need to have a high electron density. This has cast doubt on the possibility that proteoglycans could be responsible for the difference, as their high degree of hydration lowers their average electron density considerably. Proteoglycans might be responsible if their core proteins were sufficiently electron dense as these are known to be associated with collagen at the d

and e bands in the gap region. The protein or proteins responsible need not be of a particular size, and could in fact vary from species to species. This could explain one feature of X-ray diffraction patterns where a first order reflection is found e.g. in unextracted human and trout corneas. In these corneas, the collagen fibrils are narrower than in bovine, e.g. 23.5nm in trout [Gyi, 1988], compared with 37nm in bovine [Craig *et al.*, 1987], and a first order meridional reflection is usually seen on X-ray diffraction. In these cases the additional protein associated with the collagen might be particularly large compared with the narrower fibrils, leading to a higher electron density in the gap region compared with the overlap. This would account for the appearance of the first order reflection in the X- ray diffraction pattern.

4.2 Proteins extracted from bovine corneal stroma

There has been very little characterisation of the proteins extractable from bovine corneal stroma by other workers, with the exception of BCP54 [Alexander *et al.*, 1981,Silverman *et al.*, 1981], and the extractable proteoglycans [Axelsson and Heinegård, 1978,Gregory *et al.*, 1982]. Proteins removable using a mild extraction technique can be seen in figure 3.1 lane 1. BCP54 is the most prominent band present, with other major bands of M_r 45 000, 66 000 and 75 000, plus a diffuse band of staining at M_r 180 000–200 000.

The effect of the presence of βmercaptoethanol in the extraction solution can be seen in figure 3.1 lane 2 (page 54). The types and amounts of proteins extracted is virtually identical to those of lane 1, with the addition of two proteins running slightly ahead of the M_r 66 000 band. βmercaptoethanol is a reducing agent which breaks inter- and intra-polypeptide disulphide crosslinks, therefore any protein components which are covalently linked to the insoluble matrix in this way should be released and subsequently extracted in its presence. Proteins would not be expected to be covalently

linked directly to the type I collagen of the matrix via disulphide bonds, as type I collagen does not contain any cysteine residues. However, the possibility exists of indirect linkage to the framework via another protein. The two additional proteins seen in this extract are therefore likely to be associated with the insoluble framework in this way.

When SDS is also included in the extracting solution (figure 3.1 lane 3) this should remove any proteins which are strongly electrostatically bound to the insoluble matrix, and also help to remove proteins freed by the presence of βmercaptoethanol but which are not soluble in 0.15M NaCl. This extraction is sequential to that of NaCl and βmercaptoethanol, hence the reduced amounts of each of the protein bands which were present in the first extract. A new protein is present in this extract, of M_r 135 000, plus smaller amounts of two high M_r ($\sim$ 200 000) proteins. The inclusion of SDS in the extraction medium, either on its own or in conjunction with βmercaptoethanol, has therefore removed these proteins from the insoluble matrix.

In order to decide whether a reducing agent was necessary to extract the M_r 135 000 protein, corneas were extracted using NaCl plus SDS, in the presence and absence of βmercaptoethanol, as shown in figure 3.2 (page 56). The M_r 135 000 protein is definitely present in both extracts, as are the two proteins of $M_r \sim 200\,000$, and in very similar amounts, indicating that the protein is strongly electrostatically bound to the insoluble framework, but is not linked covalently to it via disulphide bonding. Thus there is no specific need for βmercaptoethanol in the extraction solutions to remove this protein; a chaotropic agent such as SDS is sufficient.

Corneas were extracted using NaCl or NaCl plus SDS for different time periods, in order to see whether there was any qualitative difference in the proteins extracted after different lengths of time.

Figure 3.3 (page 57) shows the pattern of proteins extracted after 1,3 and 5 days. The extractions were sequential, hence the reduction in the amount of proteins present in the extracts after longer periods. The proteins present in the NaCl extracts (lanes 2,4 and 6) compare well with those

of figure 3.1 lane 1, and those of NaCl plus SDS (lanes 3,5 and 7) with figures 3.1 lane 3 and 3.2 lanes 1 and 2. In the NaCl plus SDS extract, the proteins removed can all be seen in the first extract, with diminishing amounts present after longer time periods. In the NaCl extracts, most of the proteins can be seen to be in diminishing amounts, with the exception of the M_r 135 000 band. This seems to be present in increasing amounts with increased time, suggesting that it may be slightly soluble in 0.15M NaCl and can be removed to some extent with longer extraction times.

The presence of protease inhibitors in the extraction solutions should ensure that the proteins seen in the extracts are not degradation products of proteolytic or bacterial action.

Figure 3.4 (page 59) is the corresponding gel to that shown in figure 3.3 but stained for neutral sugar using periodic acid-Schiffs reagent. As presented in the results section, the M_r 135 000 band (GP135) has stained, and also the part of the gel corresponding to the diffuse area of protein staining of M_r 180 000–200 000, indicating that these both contain attached neutral sugars and are thus glycoprotein in nature.

Figure 3.5 (page 60) is the corresponding gel to that shown in figure 3.3 but stained for glycosaminoglycan using Alcian blue. Two areas of staining are present in lanes 2–7, one of which corresponds to the diffuse area of protein staining of M_r 180 000–200 000. The second area, of higher M_r, is again diffuse, but it is not possible to estimate its M_r range as it is well above the position of the highest molecular weight marker. The diffuse area of protein staining at M_r 180 000–200 000 is thus identifiable as proteoglycan which also contains attached neutral sugars. The area of staining of higher M_r is not apparent in either figure 3.3 or figure 3.4, which suggests that it could be a pure glycosaminoglycan. One possible candidate for a connective tissue would be hyaluronic acid. To date there have been no reports of hyaluronic acid in bovine cornea, although it is possible that this is an unusual form which may not have been detected. It has been noted that proteoglycans containing attached DS/CS do not stain for protein on polyacrylamide gels, suggesting that this area of stain is also pro-

teoglycan, containing attached DS/CS chains. This would imply that the 180 000–200 000 proteoglycan contains attached KS chains, and not DS/CS, however, definitive tests would be necessary to confirm this.

The very high molecular weight proteoglycans seen in the stacking gel in figure 3.5 are potentially of great interest, as they suggest the presence of previously unknown large proteoglycans in the cornea. In electron micrographs of normal bovine cornea, occasional large proteoglycan filaments can be seen (figure 4.2) which until now have been assumed to be aggregates of small proteoglycans. However, if the proteoglycans seen here are distinct large proteoglycans, which in a dissociating gel system they would be expected to be, this might suggest a separate identity for the large filaments seen in electron micrographs. This could be of major importance in certain diseases of the cornea, such as macular dystrophy, where there is abnormal deposition of large numbers of these large proteoglycan filaments. Also, in the healing of scar tissue in cornea it is well known that abnormally large proteoglycans are present [Hassell *et al.*, 1983].

These large proteogycans in cornea may have escaped detection as a result of the methods used to extract proteoglycans from the tissue. These have generally been adapted from cartilage studies, where a chaotropic agent such as 4M guanidinium chloride is used. Results here clearly show that the presence of a chaotropic agent (in this case SDS) appears to prevent removal of the proteoglycan from the tissue, whereas extraction in 0.15M NaCl facilitates its removal. This effect may result from the tissue disorganisation caused by chaotropic agents, which could conceivably trap very large proteoglycans and prevent their removal; this may not be such a problem in cartilage which has a more open structure than cornea, thus allowing large molecules to escape more easily. The swelling which occurs in 0.15M NaCl results in expansion of the corneal structure, which is not so disrupted, and would therefore allow larger molecules to escape.

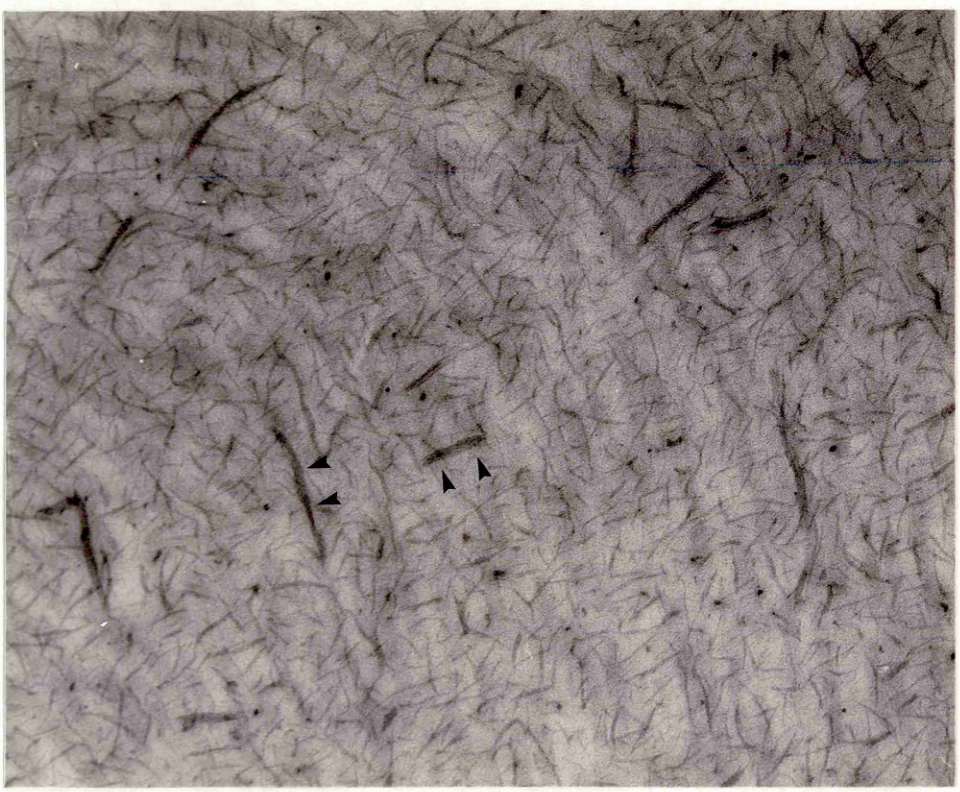


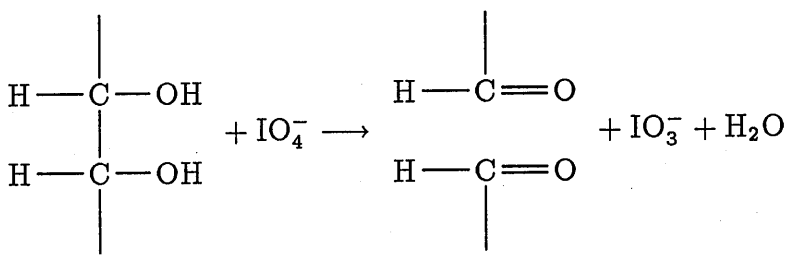
Figure 4.2 Electron micrograph of normal bovine cornea stained for proteoglycan using cupromeronic blue. Numerous small proteoglycan filaments can be seen, plus occasional large proteoglycan filaments (arrowed). Bar=200nm
Micrograph courtesy of Dr. K.M. Meek

4.2.1 The mechanism of staining of neutral sugars and glycosaminoglycans in polyacrylamide gels

Neutral sugars

The Periodic acid/Schiff staining procedure allows detection of neutral sugars by a two-stage staining method.

1. Periodic acid is used to oxidise hydroxyl groups situated on adjacent carbon atoms.



2. Schiffs reagent is added in its reduced, colourless form; this reacts with aldehyde groups produced by the periodate step. The addition product thus formed is deep pink in colour.

After the oxidation step, it is necessary to completely wash out the excess periodate from the gel, otherwise when Schiffs reagent is added this will be oxidised and the whole of the gel will turn pink.

In theory, this procedure might be expected to stain the glucuronic/iduronic acid moieties of chondroitin/dermatan sulphate, as these contain hydroxyl groups on adjacent carbon atoms. However, the close proximity of negatively charged carboxyl and sulphate groups electrostatically repels the periodate ion [Pearse, 1985]. This slows the reaction down to such a large extent that only negligible amounts of aldehyde are produced during the oxidation step, therefore there is no significant reaction with Schiffs reagent.

Glycosaminoglycans

Alcian blue is a cationic dye which specifically stains polyanions [Scott, 1972], such as the negatively charged glycosaminoglycan chains of the proteoglycans. Figure 4.3 shows a diagrammatic representation of the interaction between polyanion and the cationic dye complex.

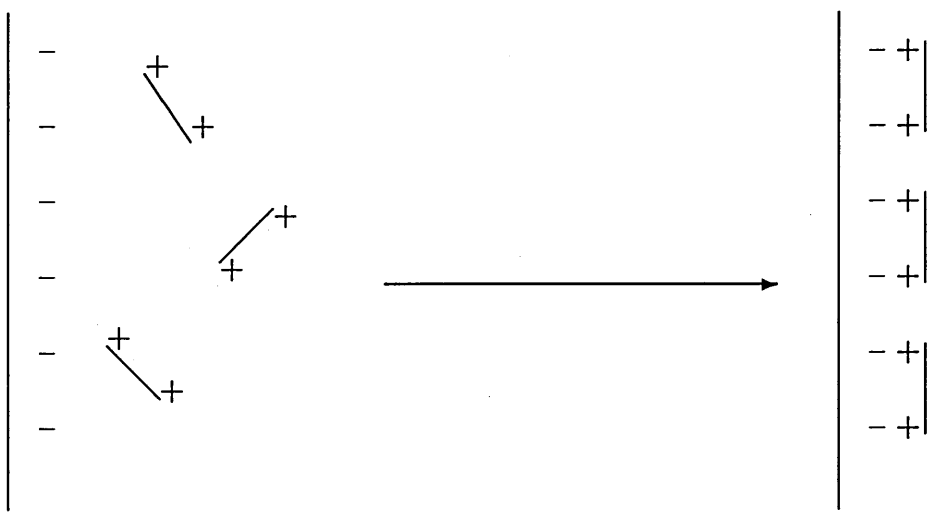


Figure 4.3 Diagram of the interaction between Alcian blue dye molecules and polyanionic species such as glycosaminoglycans

4.2.2 Refinement of Alcian blue staining procedure

As discussed above, Alcian blue stains negatively charged polyanionic species, including glycosaminoglycans. The related dyes Cuprolinic blue and Cupromeronic blue have been developed for more specific staining of glycosaminoglycans, and have been used in X-ray and electron microscopic studies in order to locate proteoglycans in various tissues, e.g. tendon [Meek *et al.*, 1985], cornea [Scott and Haigh, 1985], sclera [Young, 1985]. In biochemical studies, Alcian blue has been used in cellulose acetate electrophoresis for detecting and quantifying isolated glycosaminoglycans [Newton *et al.*, 1974], and in polyacrylamide gel electrophoresis for staining

neutral glycoproteins using a variant of the PAS method described above [Wardi and Michos, 1972].

During the course of this work, it was necessary to identify glycosaminoglycans in the components extracted from corneas and separated on polyacrylamide gels. Figure 3.6 (page 63) demonstrates the results seen in early attempts at staining using Alcian blue or Toluidine blue. Very high background staining was produced so that it was difficult to see where true staining of the components had taken place. This effect is partly due to variability in the gel polymerisation process which can lead to acrylic acid formation as part of the gel matrix, this produces areas within the gel containing acrylate anions which are effectively polyanions. These polyanionic areas thus take up stain producing a dark, patchy background against which it is impossible to discern genuine band staining. In addition, two of the standards in figure 3.6 have taken up stain; as these do not contain attached glycosaminoglycans, a lack of specificity of staining is indicated.

Polyanion	CECs in $MgCl_2$ solution (Molarities)
Hyaluronate	0.05
Polyacrylate	0.05–0.1
DNA	0.1–0.15
Chondroitin-4-sulphate	0.4
Dermatan sulphate	0.4
Chondroitin-6-sulphate	0.5
Heparan sulphate	0.4
Heparin	0.5–0.6
Keratan sulphate	0.6

Table 4.1 Critical electrolyte concentrations of some Alcian blue-polyanionic complexes on filter paper. Values taken from Scott, 1972, the dye used was Alcian blue 8GX.

In order to increase the specificity of staining, $MgCl_2$ was added to the Alcian blue stain and destain solutions [Scott, 1972]. In theory this eliminates uptake of stain by different polyanionic species depending on their 'critical electrolyte concentration', which is the concentration of $MgCl_2$

above which they cease to stain. The basis of this increase in specificity lies in the fact that staining of polyanions by Alcian blue can be inhibited by adding a competing cation, in this case Mg^{2+}. At low concentrations of Mg^{2+}, a large number of polyanionic species take up stain, however, as the Mg^{2+} concentration is increased, species with a lower affinity for the stain cease taking it up, as the Mg^{2+} effectively competes with the stain for binding sites on the polyanion. The Mg^{2+} concentration at which this occurs is known as the 'critical electrolyte concentration' (CEC). Selectivity of staining can therefore be altered by choosing a specific concentration of $MgCl_2$ in the stain and destain solutions. Table 4.1 shows the critical electrolyte concentrations of some important polyanions.

Figure 3.7 (page 64) illustrates the effect of increasing $MgCl_2$ concentration upon gel staining. Where no $MgCl_2$ was present (lanes 1 and 2) glycosaminoglycan staining can be seen at the top of the gel, but the background staining is high. Where 0.2M $MgCl_2$ is added (lanes 3 and 4) glycosaminoglycan staining is again seen at the top of the gel, but in this case background staining is greatly reduced, hence Alcian blue uptake by the gel matrix itself has been prevented. At this $MgCl_2$ concentration staining of both chondroitin sulphate and keratan sulphate should occur. Where 0.8M $MgCl_2$ is added (lanes 5 and 6) the staining pattern is very similar to that of lanes 3 and 4. This is surprising in view of the fact that both keratan sulphate and chondroitin sulphate should have ceased to stain, as this concentration of $MgCl_2$ is above both their critical electrolyte concentrations. The original CEC values were obtained for isolated glycosaminoglycans on filter paper [Scott, 1972], possibly their attachment to protein core in a polyacryamide gel matrix increases their CEC values. It may be of interest in future work to use even higher concentrations of $MgCl_2$ to see if staining can be suppressed.

Where no $MgCl_2$ is present (figure 3.7 lanes 1 and 2) there is uptake of stain by the 54 000 M_r protein band (BCP 54), this staining ceases on addition of $MgCl_2$. Uptake of the stain by this protein indicates the presence of polyanions, but in this case they are unlikely to be glycosaminoglycans;

firstly, staining ceases by 0.2M $MgCl_2$ where keratan sulphate and chondroitin sulphate would still be expected to take up stain and secondly, the protein migrates as a sharp band, whereas proteoglycans are generally polydisperse in nature migrating as diffuse bands. The staining could be accounted for by the fact that BCP 54 contains a high proportion of acidic amino acids (see table 1.3), these could be present in the protein in such a way that they would give rise to polyanionic areas on the protein which would take up stain.

In order to detect proteoglycans in various types of corneal extract a staining method based on the critical electrolyte concentration method was devised [Wall and Gyi, 1988], using Alcian blue plus 0.05M $MgCl_2$, which allows for staining of all commonly known glycosaminoglycans. Using this method, as previously discussed, background staining of the gel is reduced and non-specific staining of protein bands is eliminated.

4.2.3 Proteins extracted from normal and keratoconus human corneas

Keratoconus is a corneal disease characterised by cone-shaped ectasia of the cornea, and thinning of its central portion. At later stages of the disease this results in severe astigmatism,scarring and thus loss of vision, and in extreme cases tearing of the cornea may occur. Comparative studies of corneal collagens in normal and keratoconus corneas indicate that there is no difference in composition or distribution [Zimmermann *et al.*, 1988], thus there are probably other factors involved in the cause of the disease. Extractable proteins from normal and keratoconus human corneas were compared, in order to see if there were any differences which might lead to a further understanding of the disease. There were no major differences in the protein profiles seen on gel electrophoresis, either using mild or harsh extraction techniques (figure 3.8 page 66) with the exception of two proteins, one a small protein running just behind the dye front in normal but not keratoconus extracts, and a protein band of M_r 43 000 present in keratoconus but not normal extracts. Further work is necessary to decide the

significance of these two proteins in the development of the disease. Similarly, polyacrylamide gels stained for proteoglycan showed no significant differences in the bands present (figure 3.9 page 67). The underlying basis for this disease does not therefore appear to involve any gross changes in the extractable proteins or proteoglycans present in the corneal stroma.

4.2.4 Preparative gel electrophoresis of GP135 and BCP54

Preparative gel electrophoresis is a good method for isolating small samples of pure protein for subsequent analysis and use in the raising of specific antibodies. For amino acid analysis the protein is eluted from the gel section, dialysed against acetic acid in order to remove any residual glycine present, and then hydrolysed for amino acid analysis in the usual way.

When raising antibodies it is important to have a pure preparation of the protein of interest, in order to minimise the production of non-specific antibodies. For this reason, gels which had streaked during running were discarded, as the proteins of interest would have been contaminated with other proteins. This streaking occurred most often when gels without sample combs were used, therefore in later runs gels were prepared with sample combs and all lanes used for running extract.

In order to locate proteins after electrophoresis, a strip of gel was stained and destained, and then lined up with the original gel, in order to decide which area of gel contained the band of interest. This method had problems in that staining and destaining of the strip caused swelling of the gel so that it was slightly larger than the remaining gel, thus after lining up the two pieces it was difficult to tell if the correct area of gel had been excised. For this reason some gels were lightly stained and destained prior to excision, so that it was certain that the correct bands had been removed.

This method of obtaining a sample of protein for antibody raising has both advantages and disadvantages. Advantages include ease of preparation of a pure protein sample, whose characteristics on polyacrylamide gel electrophoresis are known; also, the polyacrylamide gel acts as an adju-

vant and so assists in the raising of antibodies. One disadvantage of this method lies in the fact that antibodies are raised to proteins which have been denatured by the treatment necessary for electrophoresis. It is possible that denatured proteins regain their native conformation once the denaturing chemicals are removed, but this depends on the particular protein involved. If the protein was originally a subunit of a larger complex it would be unlikely to regain its native conformation. However, it was hoped that a selection of the antibodies would be raised to the primary structure of the protein, and would subsequently be able to recognise it in its native conformation in further studies such as immunoelectron microscopy.

Unfortunately, western blotting and subsequent testing of the antibodies raised during the course of this work showed a low concentration of non-specific antibodies (figures 3.12 page 75 and 3.13 page 76). One possible reason for this could be that corneal proteins are not particularly antigenic (hence the success of corneal grafting operations), although this is surprising in the case of GP135, which has neutral sugar attached. The presence of sugar would normally be expected to increase a proteins antigenicity quite considerably. In future work it may be better to prepare pure samples of the proteins by methods other than gel electrophoresis, in order to raise antibodies to the proteins in their native state.

4.2.5 Further biochemical investigations of GP135

GP135 was the most prominent additional protein removed when harsh extraction methods were used, indicating that it is a structural glycoprotein, either associated with the main collagen fibril framework, or possibly with a separate structural network in the stroma. GP135 is unlikely to be related to most of the known collagen types, as its molecular weight is different from the majority of their subunits. However, it was noted that its M_r was similar to that of one of the polypeptide chains of type VI collagen, of M_r 140 000; this is extracted from cornea using similar methods to those used to extract GP135, and their molecular weights are close enough to each other within the limits of experimental error on gel electrophoresis to have

come from the same polypeptide chain. In addition, both GP135 and the 140 000 M_r chain of type VI collagen stain for neutral glycoprotein using PAS. Further tests were performed to ascertain whether GP135 was related to type VI collagen, or any other structural glycoprotein which has been studied.

One characteristic test for type VI collagen is the appearance of certain peptides following pepsin digestion, of M_r 35 000, 45 000 and 50 000 [Ayad *et al.*, 1985]. Following pepsin digestion of whole cornea, samples of the digest were run on polyacrylamide gels in order to see if the characteristic type VI peptides could be detected (figure 3.11). Type I collagen was clearly present in this extract, but no type VI peptides were seen, which is surprising in view of the fact that type VI collagen is considered a major collagenous component of the stroma [Winterhalter, 1988]. It is possible that type VI peptides were present but not detected because of the large amount of type I collagen present.

Amino acid analysis of GP135 was performed, in order to compare its composition with that of the 140 000 chain of type VI collagen, and other structural glycoproteins which have been characterised. The structural glycoprotein keratonectin isolated by Robert and coworkers is not comparable, as its M_r is only 34 000. As noted in the introduction, there is a structural glycoprotein in cornea, isolated by Alper of M_r 137 000; table 4.2 shows the composition of GP135, compared with that of the 140 000 chain of type VI collagen and the 137 000 M_r glycoprotein isolated by Alper, (1983). The compositions of these glycoproteins are broadly similar, but not close enough for any of them to be considered identical.

4.3 X-ray diffraction

Figure 3.18 (page 87) shows the X-ray diffraction patterns from corneas which were extracted using NaCl plus βmercaptoethanol or NaCl plus βmercaptoethanol plus SDS. The exposure times were short in order to record the presence or absence of the first order reflection. In figure 3.18a,

	M_r 135 000 glycoprotein (GP135)(†)	140 000 chain of type VI collagen(‡)	137 000 M_r glycoprotein(§)
Asp	94	117	72.0
Glu	116	119	127.2
Hypro	7	31	17.4
Ser	80	55	67.0
Thr	44	38	41.2
Gly	202	171	203.8
Ala	109	70	63.7
Arg	65	63	60.0
Pro	29	68	47.8
Val	44	32	68.1
Met	6	9	10.0
Ile	22	23	38.2
Leu	65	67	68.3
Phe	25	35	32.6
Hylys	not determined	21	14.1
Lys	36	32	36.7
His	44	26	11.8
Tyr	12	23	16.4
Cys	not determined	not determined	3.7

Table 4.2 Comparison of the amino acid analyses of three different structural glycoproteins.
†Presented in results section and in Wall *et al.*, (1988)
‡From Trüeb and Winterhalter, (1986)
§From Alper, (1983)
Analyses presented as residues/1000 residues

which shows the diffraction pattern from corneas extracted using NaCl plus βmercaptoethanol, there is no sign of a first order meridional reflection. This indicates that the axial electron density of the collagen has not been altered sufficiently by the extraction to produce the gap/overlap electron density step which is necessary to give a first order reflection. Figure 3.18b, taken using corneas extracted in NaCl, βmercaptoethanol and SDS, does show a first order reflection, thus enough material has been removed from the gap region to effect a change in the diffraction pattern.

Further analysis of the meridional X-ray pattern allows computation of the axial electron density along the collagen fibrils (figure 3.20), and this can be compared with the electron density profile from tendon and unextracted cornea. The profile from extracted cornea appears to be intermediate between that of unextracted cornea and tendon. It is clear that the amount of material associated with the gap region has been reduced, and there is also some change in the overlap region, where this now looks very similar to that of tendon.

Correlation with the material present in the stromal extracts indicated that GP135 was the most prominent new component to appear when the first order meridional reflection became apparent, hence the suggestion that this might be one of the components responsible for blocking the gap region [Wall *et al.*, 1988]. However, the possibility cannot be ruled out that it is the removal of one of the other components in the extract which is responsible, for instance one of the proteoglycans, or even that these may work in conjunction to block the gap region.

One major function of material blocking the gap region is in the prevention of mineralisation which occurs in collagenous tissues which calcify, such as bone and some types of tendon. Where calcification does occur, it has been shown that mineral deposits periodically in the gap regions of the collagen fibrils [White *et al.*, 1977]; in tissues which do not calcify the presence of blocking material at the gap region prevents this process.

4.4 Electron microscopy of extracted corneas

The electron micrographs shown in figures 3.14,3.15 and 3.16 (pages 79,80 and 82) illustrate the effect of increasingly harsh extraction methods on the fine structure of the corneal stroma.

As can be seen in figure 3.15, extraction using NaCl alone produces mild disruption of the structure. The collagen fibrils are no longer evenly spaced from each other as they are in untreated tissue, suggesting that one or more matrix components responsible for regulation of the fibril spacing have been removed from the structure. The fine network of material seen in the interfibrillar spaces in untreated cornea is no longer apparent in NaCl-extracted cornea, suggesting that this too has been removed by the extraction procedure. It is not possible to say whether the fine network is responsible for the maintenance of spacing in untreated cornea. The centres of a large number of fibrils cut in transverse section have not taken up stain, suggesting that the core of the fibrils might be composed of material other than collagen, which can be extracted by NaCl treatment, although it remains a possibility that this is an artifact of the processing necessary for electron microscopy. The axial banding pattern is similar to that of untreated cornea, indicating little or no change to the external structure of the collagen fibrils.

Figure 3.16, where SDS is included in the extracting solution, indicates much more severe disruption of the fine structure of the stroma. There is now marked aggregation of the fibrils, as seen in both transverse and longitudinal sections, suggesting a more complete removal of components responsible for fibril spacing by this method. The presence of βmercaptoethanol to reduce any large disulphide bonded molecules, and SDS to solubilise any normally insoluble material would assist in this. The axial banding pattern is now much more distinctive, suggesting that there has been a change in the external structure of the fibrils in this case, with removal of material over the collagen now allowing stain better access to the surface of the fibrils. At this resolution, it is difficult to tell where material has been removed from, with respect to the axial banding pattern. Also, where

collagen fibrils have clumped together, the banding pattern can be seen to be in register. It would seem likely that this process is governed by the same charge interactions which align the collagen molecules in their staggered arrangement when they first form fibrils. Thus, removal of material coating the fibrils now allows the same interactions to take place between them.

It is possible, in the case of the NaCl extracted cornea, that some of the disruption seen results from the process of extracting the cornea in the NaCl solution, which inevitably leads to gross swelling of the cornea, followed by the removal of water to dry the cornea to its original hydration. The drying method, which involved placing the corneas in dialysis bags and dialysing against a suitable concentration of high M_r polymer, was chosen in order to produce the minimum amount of disruption on drying whilst keeping the ionic environment of the tissue constant.

4.4.1 Negative staining of corneal collagen

Previous studies have shown the variation in stain penetration along a negatively-stained corneal collagen fibril [Meek and Holmes, 1983]. Averaging the electron micrograph optical density measured over several D-periods provides a representation of the axial electron density profile for the surface of the corneal collagen fibril. Figure 3.17a (page 83) shows an electron micrograph of negatively-stained corneal collagen after NaCl, βmercaptoethanol and SDS extraction; whilst the banding pattern is not as distinctive as that of tendon (see figure 1.5 page 14), it is clearly different from that of untreated corneal collagen. Figure 3.17b shows the variation in electron micrograph optical density when untreated corneal collagen fibrils are measured. There is an underlying electron density step when moving from the overlap to the gap region, with superimposed peaks in both regions. The underlying electron density step results from smaller numbers of molecules present in the gap region where there is a space between the tail of one collagen molecule and the head of the next. Figure 3.17c is the corresponding profile for corneal collagen following extraction of the whole

cornea using SDS. The electron density step is still present, and the peaks seen in the overlap region are similar, but the three peaks of electron density in the gap region are very much reduced, showing that the extraction method has removed material associated with the gap region.

4.4.2 Immunogold staining of bovine corneal stroma sections

Immunogold labelling of electron microscopy sections is a very powerful technique for the localisation of specific components within a tissue. During the course of this work, the appearance of the first order meridional reflection in the X-ray diffraction pattern correlated with the appearance of GP135 in the stromal extracts, suggesting that GP135 might be at least one of the components responsible for blocking the gap region in corneal collagen. To confirm this, it is necessary to label specifically GP135 so that it can be located in the tissue, either using electron microscopy or X-ray diffraction. Immunogold labelling for electron microscopy was chosen, due to the technical difficulties of labelling molecules with antibodies in a relatively large block of tissue, which would be necessary for X-ray diffraction. However, the antibodies raised were not specific enough for GP135, hence it was not possible to tell whether there was periodic association of GP135 with the gap region of the collagen fibrils by this method.

4.5 Transparency measurements of extracted corneas

The results shown in table 3.3 (page 97) show that there is a decrease in transparency of cornea with increasing length of time of extraction in 0.15M NaCl, however, when 4M guanidinium chloride is introduced in the extracting solution, a considerable degree of transparency is regained. This effect would traditionally be assigned to increasing order in the system, which would allow the corneas to become more transparent. While this

may be a necessary requisite for transparency, there may be other factors in cornea which contribute to transparency or the lack of it. One of the effects of corneal swelling in 0.15M NaCl would be a change in the local conditions surrounding the collagen fibrils. As the fibrils move further away from each other their ability to buffer the surrounding matrix will decrease, thus the pH of the matrix would be expected to change in the direction of the pH of the extracting solution. In all the experiments performed here the extracting solution was unbuffered, with a pH in the region of 4.8. This is considerably lower than physiological pH, therefore a change in the charge distribution on the corneal collagen fibrils would be expected, the drop in pH causing them to become more positively charged. Highly negatively charged proteoglycans in the vicinity of the fibrils would be electrostatically attracted to the fibrils, and aggregation would occur; the more positively charged the fibrils become then the greater the expected aggregation would be, and hence decrease in transparency. This is in agreement with the work of Goodfellow (1975), who found that decreasing transparency correlated with a decrease in the pH of the bathing solutions in a range of buffered solutions at different pH.

If the above occurs, then addition of 4M guanidinium chloride to the extracting solution must have some effect on the interaction between the collagen fibrils and proteoglycans. The pH of this extracting solution is similar to NaCl alone (5.0), so the effect is not likely to be due to a change in pH, however, the large increase in ionic strength of the solution would be expected to have a considerable screening effect between the proteoglycans and collagen fibrils, and may disaggregate them sufficiently for a degree of transparency to be regained. Once this effect is removed when the guanidinium chloride solution is washed out, the proteoglycans and collagen will aggregate again, and the transparency will decrease once more.

One difficulty with this hypothesis is that proteoglycans are continuously being removed from the stroma by the extracting solution, so that while initially transparency might be expected to decrease because of aggregation, the gradual removal of more and more proteoglycans would lead

to an increase in transparency. As this does not occur it is necessary to suggest that there might be two populations of proteoglycans, one of which is more easily removed and appears in the extracting solution, the other being more strongly bound to the collagen and would then contribute to the aggregation effect.

An indication that proteoglycan aggregation does play a major role in transparency comes from the study of certain corneal diseases. In macular corneal dystrophy the collagen fibrils are of a similar size and organisation to those of normal cornea, but there are areas in the stroma containing aggregated (or possibly very large) proteoglycans [Meek *et al.*, 1989]. These corneas have areas of opacity which eventually result in the necessity of a corneal graft, thus it would seem that it is proteoglycan aggregation, and not loss of order in the corneal stroma, which has resulted in loss of transparency in this disease.

4.6 Discussion summary and suggestions for further work

Polyacrylamide gel electrophoresis of corneal extracts has provided an overall picture of the types of proteins, glycoproteins and proteoglycans extractable from corneal stroma under different conditions. Correlation with results from X-ray diffraction and electron microscopy, which both indicate removal of material from the gap region of the corneal collagen fibrils, indicates that GP135 might be one of the components associated with a large proportion of the gap regions. Whether GP135 is related to any of the other known structural glycoproteins in the cornea is not certain, although it has many features in common with type VI collagen, and it would be of interest to pursue this in further work. A particularly useful device would be the raising of suitable antibodies to GP135 to locate it precisely in the corneal stroma. Of the other components extracted from the stroma, the very high molecular weight proteoglycans seen in the stacking gel of polyacrylamide gels are definitely of interest, and should be investigated

further with respect to their attached glycosaminoglycans and the location of the proteoglycans in the tissue. It would also be of interest to further characterise the other two types of extractable proteoglycans and compare them with the proteoglycans investigated by other workers. BCP54 is of interest in the cornea because it is the major soluble protein, but as yet no suggestion has been made as to its purpose in the stroma.

X-ray diffraction provides a very accurate method for computing the axial electron density of corneal collagen fibrils in wet, unfixed tissue, therefore a method which would allow specific antibody labelling of components in whole cornea prior to X-ray diffraction would provide valuable information about the association of components with the collagen. This is currently technically difficult due to the problem of diffusing large antibody molecules into the tightly-knit structure of the stroma.

Densitometer measurements have shown that transparency of the cornea is very dependent on the type of extraction solution it is immersed in, possibly as a result of changes in the local environment of the collagen fibrils, in addition to the disorder to the system caused by swelling. *In situ* measurement of pH at different hydrations and ionic strengths of the extraction solution, and their correlation with transparency measurements might indicate whether this is an important factor in corneal transparency.

References

Abedin, M.Z., Ayad, S. and Weiss, J.B. (1982) *Biosci. Rep.* **2**, 493-502.

Alexander, R.J., Silverman, B. and Henley, W.L. (1981) *Exp. Eye Res.* **32**, 205-216.

Alper, R. (1983) *Curr. Eye Res.* **2**, 7, 479-487.

Alper, R. and Amenta, P.S. (1986) *suppl. to Invest. Ophth. and Vis. Sci.* **27**, 3, 250..

Axelsson, I. and Heinegård, D. (1978) *Biochem. J.* **169**, 517-530.

Ayad, S., Chambers, C.A., Shuttleworth, C.A. and Grant, M.E. (1985) *Biochem. J.* **230**, 465-474.

Bächinger, H.P., Doege, K.J., Petschek, J.P., Fessler, L.I. and Fessler, J.H. (1982) *J. Biol. Chem.* **257**, 14590-14592.

Benedek, G.B. (1971) *Appl. Optics* **10**, 459-473.

Birk, D.E. and Lande, M.A. (1981) *Biochim. et Biophys. Acta* **670**, 362-369.

Burgeson, R.E. (1987) In 'Structure and Function of Collagen Types' ,Eds. R. Mayne and R.E. Burgeson, pp. 145-172. Academic Press (London).

Burgeson, R.E. and Hollister, D.W. (1979) *Biochem. Biophys. Res. Commun.* **87**, 1124-1131.

Burrin, D. (1983) In 'A Biologists Guide to Principles and Techniques of Practical Biochemistry' 2nd ed., Eds. B.L. Williams and K. Wilson, pp. 277-309. Edward Arnold.

Carlson, E.C. and Waring, G.O. (1988) *Invest. Ophthalmol. Vis. Sci.* **29**, 4, 578-585.

Carrington, S.D., Alexander, R.A. and Grierson, I. (1984) *J. Anat.* **139**, 2, 319-332.

Chapman, J.A. (1974) *Connect. Tissue Res.* **2**, 137-150.

Chung, E., Rhodes, R.K. and Miller, E.J. (1976) *Biochem. Biophys. Res. Commun.* **71**, 1167-1174.

Craig, A.S. and Parry, D.A.D. (1981) *J. Ultrastruct. Res.* **74**, 232-239.

Craig, A.S., Robertson, J.G. and Parry, D.A.D. (1987) *J. Ultrastruct. and Mol. Struct. Res.* **96**, 172-175.

Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) *Biochemistry* **10**, 2606-2617.

Fatt, I. (1978) In 'The Physiology of the eye: An Introduction to the Vegetative Functions'. Butterworths (London).

Fitch, J.M., Mentzer, A., Mayne, R. and Linsenmayer, T.F. (1988) *Dev. Biol.* **128**, 396-405.

Fleischmajer, R., Timpl, R., Tuderman, L., Raisher, L., 'Wiestner, M., Perlish, J.S. and Graves, P.N. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7360-7364.

Furoto, D.K. and Miller, E.J. (1980) *J. Biol. Chem.* **255**, 290-295.

Furthmayr, H. and Timpl, R. (1971) *Anal. Biochem.* **41**, 510.

Gibson, G.J., Schor, S.L. and Grant, M.E. (1981) *Biochem. Soc. Trans.* **9**, 550-551.

Goldman, J.N. and Benedek, G.B. (1968) *Invest. Ophthal.* **6**, 574-600.

Goodfellow, J.M. (1975) Ph.D. Thesis, The Open University.

Goodfellow, J.M., Elliott, G.F. and Woolgar, A.E. (1978) *J. Mol. Biol.* **119**, 237-252.

Gregory, J.D., Cöster, L. and Damle, S.P. (1982) *J. Biol. Chem.* **257**, 12, 6965-6970.

Gyi, T.J. (1988) Ph.D. Thesis, The Open University.

Haigh, M. and Scott, J.E. (1986) *Basic Appl. Histochem.* **30**, 479-486.

Hall, C.E. and Slayter, H.S. (1959) *J. Biophys. Biochem. Cytol.* **5**, 11-17.

Hames, B.D. and Rickwood, D. (1981) In 'Gel Electrophoresis of Proteins' p.6 IRL Press (Oxford).

Harding, J., Crabbe, M.J.C. and Panjwani, N.A. (1980) *Colloques Internationaux du C.N.R.S* **287**, 51-64.

Harnisch, J.-P., Buchen, R., Sinha, P.K. and Barrach, H.J. (1978) *Graefes Archiv. klin. exp. Ophthal.* **208**, 9-13.

Hart, R.W. and Farrell, R.A. (1971) *Bull. Maths. Biophys.* **33**, 165-186.

Hassell, J.R., Cintron, C., Kublin, C. and Newsome, D.A. (1983) *Arch. Biochem. Biophys.* **222**, 362-369.

Heinegård, D., Wieslander, J., Sheehan, J., Paulsson, M. and Sommarin, Y. (1985a) *Biochem. J.* **225**, 95-106.

Heinegård, D., Björne-Persson, A. and Cöster, L. (1985b) *Biochem. J.* **230**, 181-194.

Henkel, W. and Glanville, R.W. (1982) *Eur. J. Biochem.* **122**, 205-213.

Hodge, A.J. and Petruska, J.A. (1963) In 'Aspects of Protein structure', Ed. G.W. Ramachandran. Academic Press (New York). , .

Hodge, A.J. and Schmitt, F.O. (1960) *Proc. Nat. Acad. Wash.* **46**, 186.

Holt, W.S. and Kinoshita, J.H. (1973) *Invest. Ophthalmol.* **12**, 114-126.

Huber, s., van der Rest, m., Bruckner, p., Rodriguez, E., Winterhalter, K.H. and Vaughan, L. (1986) *J. Biol. Chem.* **261**, 5965-5968.

Hulmes, D.J.S., Miller, A., White, S.W. and Doyle, B.B. (1977) *J. Mol. Biol.* **110**, 643-666.

Hulmes, D.J.S., Miller, A., White, S.W., Timmins, P.A. and Berthet-Colominas, C. (1980) Int. J. Biol. Macromol. **2**, 338-346.

Jakus, M.A. (1956) *J. Biophys. Biochem. Cytol. suppl. 2* 241-252.

Junqua, S., Menasche, M., Brechemier, D., Pouliquen, Y. and Robert, L. (1975) *Archs. Ophtal. Paris* **35**, 665-668.

Kapoor, R., Bornstein, P. and Sage, H. (1985) *Biol. Chem. Basement Membr. [Proc. Int. Symp.], 1st* 105-118.

Kefalides, N.A. (1973) *Int. Rev. Connect. Tissue Res.* **6**, 63-104.

Labermeier, U. and Kenney, M.C. (1983) *Biochem. Biophys. Res. Commun.* **116**, 619-625.

Laemmli, U.K. (1970) *Nature (London)* **227**, 680-685.

Laurain, G., Delvincourt, T. and Szymanowicz, A.G. (1980) *FEBS Lett.* **120**, 44-48.

Linsenmayer, T.F., Fitch, J.M., Schmid, T.M., Zak, N.B., Gibney, E., Sanderson, R.D. and Mayne, R. (1983) *J. Cell Biol.* **96**, 124-132.

Linsenmayer, T.F., Bruns, R.R., Mentzer, A. and Mayne, R. (1986) *Dev. Biol.* **118**, 425-431.

Linsenmayer, T.F., Gibney, E. and Little, C.D. (1982) *Exp. Eye Res.* **34**, 371-379.

Marchini, M., Moracutti, M., Ruggeri, A., Koch, M.H.J., Bigi, N., and Roveri, N. (1986) *Conn. Tiss. Res.* **15**, 269-281.

Maurice, D.M. (1969) In 'The Eye' Vol 1, Ed. H. Davson, pp.489-600. Academic Press (London).

Maurice, D.M. (1957) *J. Physiol.* **136**, 263-286.

Meek, K.M., Elliott, G.F., Sayers, Z., Whitburn, S.B. and Koch, M.H.J. (1981a) *J. Mol. Biol.* **149**, 477-488.

Meek, K.M., Elliott, G.F., Sayers, Z. and Whitburn, S.B. (1981b) *Current Eye Res.* **1**, 281-284.

Meek, K.M. and Chapman, J.A. (1985) *J. Mol. Biol.* **185**, 359-370.

Meek, K.M., Scott, J.E. and Nave, C. (1985) *J. Microscopy* **139**, 2, 205-219.

Meek, K.M. and Holmes, D.F. (1983) *Int. J. Biol. Macromol.* **5**, 17-25.

Meek, K.M., Quantock, A.J., Elliott, G.F., Ridgway, A.E.A., Tullo, A.B., Bron, A.J. and Thonar, E.J.M.A. (1989) *Exp. Eye Res.* **49**, 941-958.

Menasche, M. (1988) 8th Int. Congress of Eye Research, San Francisco, U.S.A. Workshop proceedings, (unpublished).

Mishima, S. (1968) *Survey Ophthalmol.* **13**, 57-96.

Newton, D.J., Scott, J.E. and Whiteman, P. (1974) *Anal. Biochem.* **62**, 268-273.

Pearse, A.G.E. (1985) In 'Histochemistry: Theoretical and Applied' Vol II Analytical Technology. 4th Ed. Churchill Livingstone (London).

Piez, K.A., Eigner, E.A. and Lewis, M.S. (1963) *Biochemistry* **2**, 58-66.

Pirie, A. (1951) *Biochemistry* **48**, 368-371.

Robert, L. (1986) *Front. Matrix Biol.* **11**, 1-16.

Robert, L. and Parlebas, J. (1965) *C. r. hebd. Seanc. Acad. Sci. Paris* **261**, 842-844.

Rojkind, M., Giambrone, M.A. and Biempica, L. (1979) *Gastroenterology* **76**, 710-719.

Sage, H. and Bornstein, P. (1987) In 'Structure and Function of Collagen Types' ,Eds. R. Mayne and R.E. Burgeson, pp. 173-194. Academic Press (London).

Sage, H., Crouch, E. and Bornstein, P. (1979) *Biochemistry* **18**, 5433-5422.

Sawada, H. (1982) *Cell Tissue Res.* **226**, 241-255.

Sayers, Z., Koch, M.H.J., Whitburn, S.B., Meek, K.M., Elliott, G.F. and Harmsen, A. (1982) *J. Mol. Biol.* **160**, 593-607.

Schittny, J.C., Timpl, R. and Engel, J. (1988) *J. Cell Biol.* **107**, 1599-1610.

Scott, J.E. (1972) *Histochemie* **32**, 191-212.

Scott, J.E. (1988) *Biochem. J.* **252**, 313-323.

Scott, J.E. and Haigh, M. (1985) *Biosci. Rep.* **5**, 765-774.

Scott, J.E. and Haigh, M. (1988) *Biochem. J.* **253**, 607-610.

Shapiro, A.L., Vinũela, E. and Maizel, J.V. (1967 *Biochem. Bipohys. Res. Comm.* **28**, 815.

Silverman, B., Alexander, R.J. and Henley, W.L. (1981) *Exp. Eye Res.* **33**, 19-29.

Smelser, G.K. (1960) In 'The Transparency of the Cornea', Eds. W.S. Duke-Elder and E.S. Perkins. Blackwell (Oxford).

Tervo, T., Sulonen, J., Valtonen, S., Vannas, A. and Virtanen, I. (1986) *Exp. Eye Res.* **42**, 399-406.

Tomlin, S.G. and Worthington, C.R. (1956) *Proc. Roy. Soc. ser. A* **235**, 189-201.

Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci.* **76**, 9, 4350-4354.

Trüeb, B. and Bornstein, P. (1984) *J. Biol. Chem.* **259**, 8597-8604.

Trüeb, B. and Winterhalter, K.H. (1986) *EMBO J.* **5**, 2815-2819.

Tzaphlidou, M. and Chapman, J.A. (1986) *Micron and Microscopica Acta* **17**, 3, 269-280.

von der Mark, H., Aumailley, M., Wick, G., Fleischmajer, R. and Timpl, R. (1984) *Eur. J. Biochem.* **142**, 493-502.

Wall, R.S. and Gyi, T.J. (1988) *Anal. Biochem.* **175**, 298-299.

Wall, R.S., Elliott, G.F., Gyi, T.J., Meek, K.M. and Branford-White, C.J. (1988) *Biosci. Rep.* **8**, 1, 77-83.

Wardi, A. and Michos, G. (1972) *Anal. Biochem.* **49**, 607-609.

White, S.W., Hulmes, D.J.S., Miller, A. and Timmins, P.A. (1977) *Nature* **266**, 421-425.

Winterhalter, K.H. 8th Int. Congress of Eye Research, San Francisco, U.S.A. Workshop proceedings, (unpublished).

Worthington, C.R. and Inouye, H. (1985) *Int. J. Biol. Macromol.* **7**, 2-8.

Young, R.D. (1985) *J. Cell Sci.* **74**, 95-104.

Zimmermann, D.R., Trüeb, B., Winterhalter, K.H., Witmer, R. and Fischer, R.W. (1986) *FEBS Letters* **197**, 55-58.

Zimmermann, D.R., Fischer, R.W., Winterhalter, K.H., Witmer, R. and Vaughan, L. (1988) *Exp. Eye Res.* **46**, 431-442.